

Proceedings of the Society for Experimental Biology and Medicine

VOL. 95

AUGUST-SEPTEMBER, 1957

No. 4

Effect of Selenium in Preventing Exudative Diathesis in Chicks. (23307)

E. L. PATTERSON, R. MILSTREY AND E. L. R. STOKSTAD
Research Division, American Cyanamid Co., Pearl River, N. Y.

Schwarz(1) observed that rats fed a diet containing torula yeast as sole source of protein develop liver necrosis when vit. E is omitted from the diet. This liver necrosis could be prevented by vit. E, cystine or a non-fat soluble substance in vit. E-free casein or brewers yeast which he designated Factor III. Scott *et al.*(2) found that exudative diathesis is produced when chicks are fed a vit. E-free diet containing torula yeast. These workers also found that this could be prevented by alpha-tocopherol but not by gamma-tocopherol or an antioxidant such as p-diphenylphenylenediamine. Exudative diathesis developed on this torula yeast diet in the absence of added unsaturated fatty acids and the incidence of this lesion was not affected by the fat content of the diet. We have found that vit. E-free casein and several defatted pork tissues such as pancreas and kidney will also prevent exudative diathesis. Attempts have been made to isolate this factor from casein and kidney. The activity in casein and kidney was insoluble in water and alcohol. The factor in casein could be made soluble and diffusible by hydrolysis with trypsin and with sulfuric acid. Little or no purification was obtained by use of countercurrent extraction with immiscible solvents or by adsorption on activated charcoal or Magnesol. It could be adsorbed on Dowex-50 (sodium form) and eluted with 0.2 N sodium hydrox-

ide. It could also be adsorbed on Dowex-1 (chloride form) and eluted with 0.1 N hydrochloric acid. This suggests that the factor as present in acid hydrolyzed kidney is amphoteric. It was also observed that an ash of acid hydrolyzed kidney was inactive, but that an ash of kidney made alkaline with calcium oxide before burning was active. This activity of an alkaline ash and inactivity of an acid ash suggested that the activity of this factor in kidney was due to an element which forms a volatile inorganic acid. Among the elements which form volatile acids are arsenic, selenium and tellurium. Of these 3, selenium is present in the cystine and methionine fraction of wheat from seleniferous soil (3).

Methods. The basal diet used is shown in Table I. This diet, which contained 40% torula yeast and 5% of vit. E-free lard* gave the same results as the one described by Scott *et al.*(2) which contained 60% torula yeast and 5% vit. E-free lard. Day-old White Rock chicks were depleted on the basal diet 6 to 13 days, and then placed on experimental diets. Chicks on the basal diet and on basal diet plus a vit. E supplement had the same growth rate until they were 2 weeks old. The symptoms of exudative diathesis began to appear at the 18th day. The chicks were examined for lesions twice a week. Alcohol

* Purchased from Distillation Products Industries.

extracted casein was prepared by continuous extraction of crude Argentine casein with alcohol at boiling point of the solvent. Pork kidney, liver, lung, spleen and pancreas were defatted with chloroform and then dried in vacuum at 60° for 18 hours. Acid hydrolysis

TABLE I. Composition of Basal Diet.

Torula yeast	40.0 g
Cerelose	40.7
Vit. E-free lard	5.0
Calcium gluconate	5.0
Cellophane	3.0
Bone ash	2.0
Glycine	.5
DL-methionine	.4
L-arginine monohydrochloride	.24
L-cystine	.2
Salts*	2.0
Vitamin†	1.0

* Salt supplements in mg/100 g of diet were dipotassium acid phosphate 600, monopotassium acid phosphate 450, sodium chloride 600, magnesium sulfate trihydrate 250, ferric citrate 50, manganese sulfate monohydrate 40, copper sulfate pentahydrate 2, zinc acetate dihydrate 1.4, aluminum sulfate octadecahydrate 1.6, potassium bromide .8, cobalt acetate tetrahydrate .4, nickel chloride hexahydrate .2, and sodium molybdate dihydrate .05.

† Vitamin supplements in a cerelose diluent in mg/100 g of diet were meso-inositol 100, calcium pantothenate 5, niacin 5, riboflavin 1, thiamine hydrochloride 1, pyridoxine hydrochloride 1, menadione .027, folic acid .2, biotin .002, and vit. A and D, 1000 and 200 units.

of casein and kidney was carried out by digesting 100 g of casein or 500 g of fresh kidney with 1 l of 2 N sulfuric acid for 4 hours at 120°C. The hydrolysate was diluted 10 times and the sulfuric acid precipitated with calcium oxide. The filtrate was concentrated and dried *in vacuo*. Adsorption and elution with ion exchange resins were carried out as follows: Two hundred forty g acid hydrolyzed kidney were dissolved in 2400 ml of 0.1 M sodium bisulfate at pH 2. This was adsorbed on a 4" x 25" Dowex-50 (Na⁺) column. The column was eluted first with 20 l of 0.2 M sodium bisulfate at pH 2, and second with 26 l of 0.1 M sodium hydroxide. Two hundred forty g of acid hydrolyzed kidney were dissolved in 2400 ml of a solution containing 0.1 M sodium chloride and 0.02 M potassium dibasic phosphate at pH 9. This was percolated through a 4" x 20" Dowex-1 (Cl⁻) column. This was developed first with

15 l of a solution containing 0.2 N sodium chloride and 0.01 M potassium dibasic phosphate at pH 9, and then with 30 l of 0.2 N hydrochloric acid. Acid and alkaline ashes of kidney acid hydrolysate were prepared by adding 1.5 equivalents of sulfuric acid or calcium oxide to 500 g of kidney hydrolysate in 840 ml of water. The pH of the solutions were 2 and 9.5 respectively for acid and alkaline solutions. The mixtures were dried in an air oven and then ashed for 16 hours at 500°C. Selenium determinations were made by procedure described in Official Methods of Analysis(4).†

Results. The effect of a number of supplements on incidence of exudative diathesis and growth is shown in Table II. It can be seen that 40 units of tocopherol acetate‡ gave complete protection. In other experiments 12 units were adequate but 4 units were not. The results show that 50 or 100 g of casein added at 6 or 8 days gave almost complete protection against exudative diathesis. When the experiment was continued to 28 days more symptoms may have developed, showing that 100 g of casein did not give complete protection over a long period of time. An amino acid mixture simulating casein, fed at 100 g/kg, was inactive. The amino acid mixture supplied the following quantities of amino acid/kg of diet: l-arginine • HCl, 4.7 g; l-histidine • HCl, 3 g; l-lysine • HCl, 8.3 g; dl-tryptophan, 1.8 g; l-phenylalanine, 5 g; l-cystine, 0.4 g; dl-methionine, 3.6 g; dl-serine, 15.3 g; l-threonine, 4 g; l-leucine, 12 g; l-isoleucine, 6.6 g; dl-valine, 14 g; l-glutamic acid, 33 g; l-aspartic acid, 6.3 g; l-glycine, 0.5 g; l-alanine, 5.6 g; l-hydroxyproline, 2 g; l-proline, 8.3 g and l-tyrosine, 6.3 g.

A number of defatted pork tissues were assayed and the results, given in Exp. III of Table II, show that pork kidney and pancreas were the most active, and heart the least. A commercial acid hydrolysate of

† We are indebted to Mr. W. C. Groth for making selenium analyses.

‡ Supplied as Myvamix, commercial preparation made by Distillation Products, Inc. containing 44 units/g of vit. E activity as tocopherol acetate.

TABLE II. Effect of Various Substances on Incidence of Exudative Diathesis in Chicks.

Exp. No. and time of de- pletion, days	Supplement/kg diet	No. of chicks started	Wt at 23 days and (survivors)	No. of chicks showing exudative diathesis
I 7	None	11	137 (9)	7
	40 units tocopherol acetate	11	195 (11)	0
II 7	None	9	157 (6)	6
	50 g alcohol extr. casein	9	214 (7)	1
	100 g amino acid mix	6	146 (6)	5
	100 g Sheffield acid hydr. casein + 4 g tryptophan	9	175 (9)	2
III* 13	None	12	203 (12)	9
	50 g alcohol extr. casein	12	231 (11)	6
	100 <i>Idem</i>	12	242 (12)	4
	30 g defatted hog liver	12	253 (12)	0
	30 heart	12	227 (11)	9
	30 brain	12	235 (12)	2
	30 kidney	12	242 (11)	0
	30 lung	12	250 (12)	0
	30 pancreas	12	238 (12)	0
	30 spleen	12	236 (12)	1
IV 13	None	10	161 (4)	6
	10 g acid hydr. kidney	10	200 (10)	3
	30 <i>Idem</i>	10	219 (10)	0
	Acid ash equiv. to 44 g kidney	10	169 (5)	8
	Alkaline ash equiv. to 41 g kidney	10	213 (10)	2
V 13	None	11	190 (6)	11
	15 g acid hydr. kidney	11	241 (11)	0
	Acid ash equiv. to 80 g kidney	11	197 (11)	8
	Alkaline ash equiv. to 80 g kidney	11	235 (11)	0
VI 13	None	11	170 (5)	11
	45 g acid hydr. kidney	11	240 (11)	2
	Acid filtrate and washings of Dowex-50 column equiv. to 40 g kidney hydr.	11	180 (2)	9
	Alkaline eluate of Dowex-50 column equiv. to 40 g of kidney hydr.	11	256 (10)	0
	Alkaline filtrate and washings of Dowex-1 column equiv. to 40 g kidney hydr.	11	200 (6)	10
	Acid eluate of Dowex-1 column equiv. to 40 g of kidney hydr.	11	227 (11)	1
VII 13	None	12	192 (10)	11
	15 g acid hydr. kidney	12	240 (10)	1
	1 ppm selenium as selenite	12	260 (10)	1
	10 <i>Idem</i>	12	250 (11)	0
	10 arsenic as equal mixture of arsenate and arsenite	12	217 (9)	10
VIII 13	None	12	164 (8)	10
	10 g acid hydr. kidney	12	223 (8)	5
	20 <i>Idem</i>	12	259 (12)	1
	.3 ppm selenium as selenite	12	252 (12)	0
	1 <i>Idem</i>	12	245 (12)	0
	3 "	12	257 (12)	0
	3 tellurium as tellurite	12	222 (12)	4

* Experiment ran 22 days instead of 23 days.

casein[§] supplemented with tryptophan was somewhat less active than native crude

casein. A sulfuric acid hydrolysate of kidney proved the best source of the factor for fractionation work. The results of Exp. IV in Table II showed that 30 g of this were required for complete protection. The factor

[§] A sulfuric acid hydrolysate of casein made by Sheffield Co.

in hydrolyzed kidney was adsorbed on Dowex-50 (Na^+) and eluted by dilute sodium hydroxide. Similarly the activity was also adsorbed on and eluted from the anion exchange Dowex-1. The assay results of Exp. VI on these ion exchange fractions show that the active principle is amphoteric.

The alkaline ash of acid hydrolyzed kidney was active but it is apparent that some activity was lost during the ashing process. The acid ash was less active. The alkaline ash contained 1.8 ppm of selenium and the acid ash contained 1 ppm of selenium. The original hydrolyzed kidney contained 3.5 ppm of selenium. Hydrolyzed kidney yielded 37% of alkaline ash, so approximately 20% of the selenium was recovered during the ashing process. Seven % of the selenium was recovered in the acid ash.

The activity of selenium and tellurium is shown in Exp. VII and VIII. Selenium gave complete protection in Exp. VIII at 0.3 and 1 ppm. Tellurium as tellurite was inactive at 3 ppm. Arsenic showed no activity at 10 ppm.

Discussion. It is apparent from these data that selenium can replace vit. E for prevention of exudative diathesis on chicks on a torula yeast diet. It is not possible to state whether the need for selenium can be completely eliminated by vit. E. The torula yeast in the diet contained less than 0.1 ppm of selenium which would contribute less than 0.04 ppm to the basal diet. Addition of vit. E to this diet gave good growth with no evidence of exudative diathesis at 35 days. If selenium is required in the presence of vit. E the requirement would appear to be less than 0.04 ppm.

Selenium is toxic for many species of animals such as the horse, cow, pig, dog and chicken(5). Poley *et al.*(6) reported that the minimum level of selenium which would depress growth rate of chicks was 10 ppm when given in the form of seleniferous grains. These same workers(7) also found that levels in excess of 5 ppm reduced hatchability and that 10 ppm of selenium decreased it to zero.

There is no convincing published evidence that selenium is necessary for animal nutri-

tion. Poley *et al.*(6) stated that chicks receiving diets containing 5 ppm of selenium in seleniferous grains usually grew better than those receiving none or 2 ppm. In view of the fact that 1 ppm of selenium as selenite is adequate and that Poley's diets contained vit. E in the natural grains used, it seems unlikely that the better growth reported with 5 ppm was due to the additional selenium. Pinsent(8) found that 3 to 8 μg of selenium/ml was necessary for the formation of formic dehydrogenase by *Escherichia coli*. This suggests a role of selenium for certain phases of bacterial metabolism. Very recently, Schwarz and Foltz(9) have found that selenium is protective against dietary necrotic liver degeneration in rats that are deficient in "factor 3."

Summary. (1) Chicks fed a diet containing torula yeast developed exudative diathesis which could be prevented by either vit. E or a non-fat soluble substance in casein and in a number of pork tissues. (2) This factor could be made water soluble by acid hydrolysis. It was adsorbed on both anion and cation exchange resins and thus behaved as an ampholyte. (3) An alkaline ash but not an acid ash of pork kidney was effective in preventing exudative diathesis. (4) Selenium as selenite prevented exudative diathesis at 0.3 ppm. Tellurium as tellurite was ineffective at 3 ppm. Anionic arsenic at 10 ppm was inactive.

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Received May 21, 1957. P.S.E.B.M., 1957, v95.

Prevention of Exudative Diathesis in Chicks by Factor 3 and Selenium. (23308)

KLAUS SCHWARZ, JOHN G. BIERI, GEORGE M. BRIGGS AND MILTON L. SCOTT

National Institute of Arthritis and Metabolic Diseases, N.I.H., Bethesda, Md., and Department of Poultry Husbandry, Cornell University, Ithaca, N. Y.

Factor 3 is a dietary agent preventing necrotic liver degeneration in the rat(1) and multiple degeneration (heart, liver, kidney and muscle necrosis) in the mouse(2). Recently, Factor 3 has been identified as an organic compound containing selenium, and selenite was found to replace it in the diet (3).^{*} The present study shows that Factor 3 preparations of greatly differing degrees of purity, as well as selenium in organic and inorganic forms, prevent exudative diathesis in the chick when applied at dose levels similar to those effective against liver necrosis in the rat. The data presented here result from collaborative experiments carried out since 1955 at the National Institutes of Health and at Cornell University.

The production of necrotic liver degeneration in the rat, using a vit. E and Factor 3-free, purified ration based on 30% *Torula* yeast as the source of protein, has been described(4). Scott has adapted this diet to the amino acid requirements of the chick and has found that it produces exudative diathesis, inhibition of growth, and death, whereas the liver remains macroscopically normal(5). Exudative diathesis has been noted before to be produced in chicks only by certain types of vit. E-free diets, and not by others(6). The deficiency symptoms in the chick on *Torula* yeast rations were preventable not only by tocopherol but by brewers yeast as well(5). The latter is known to be free from vit. E but it is an effective source of Factor 3(7).

Methods. The Factor 3 preparations were obtained in the course of a project aimed at

purification and identification of the active principle from dried, defatted pork kidney powder.[†] Three fractions of different degrees of concentration were submitted to tests in the chick. Factor 3 activity was determined by prophylactic assay against necrotic liver degeneration using the 30% *Torula* yeast diet and the Fisher strain of rats. Values reported for Factor 3 (Table I) are the result of repeated assays with 10 rats per group. They are expressed in units, 1 unit representing the amount of Factor 3 required daily per rat to afford 50% protection(8). For complete protection against liver necrosis, the Fisher strain of rats requires ca 65 units of Factor 3 in 100 g of diet.

Selenium was tested in 3 different forms: (a) organically bound as seleno-cystathionine; this material was isolated from seleniferous grain by Horn and Breese Jones(9) as an isomorphous compound containing 20.6% selenium,[‡] (b) as sodium selenite (Foote Mineral Co.), and (c) as elementary selenium (gray modification). The first 2 materials were supplemented to the rat and chick diets as solutions in water. The elementary selenium was added as a trituration in lactose.

The chick experiments were carried out as follows: At the Nat. Inst. of Health, New Hampshire chicks, 6 or 7 per group, were fed basal diet C34, which is only slightly different from the Cornell diet. It contains *Torula* yeast 60%, cerelose 28.5%, salts 6%(10), vit. E-free lard 4%, glycine 1%, methionine 0.38%, and vitamins(10) except E. At Cornell University, White Plymouth Rock chicks, 17-20 per group, were fed the previously described basal diet A(5) containing *Torula* yeast 58.5%, cerelose 28.3%, salts 4.2%,

^{*} Since various forms of selenium show different biological activities, the term "Factor 3" is used to designate the biologically active, selenium-containing component, or components, present in nutrients and other materials of biological origin. Factor 3 is bound to protein, stable against acid hydrolysis, and water soluble(15).

[†] Schwarz, K. and others, to be published separately.

[‡] The authors wish to thank Dr. M. J. Horn for a generous sample of this material.

FACTOR 3 AND SELENIUM IN EXUDATIVE DIATHESIS

TABLE I. Prevention of Exudative Diathesis by Factor 3 and Selenium in Different Forms.

Diet treatment	Dose per 100 g of diet	Units of Factor 3* per 100 g of diet	Nat. Inst. Health			Cornell Univ.		
			Avg wt, 3 wk (g)	No. of chicks	Incidence exudative diathesis	Avg wt, 3 wk (g)	No. of chicks	Incidence exudative diathesis
Basal			164	18†	13	116	17	17
+ d α -Tocopheryl acetate	1.6 mg					164	17	2
+ <i>Idem</i>	2.4 "					143	17	1
+ d, l- α -Tocopheryl acetate	10 "							
+ Dried brewers yeast†	10 g	105	263	6	0			
+ Casein, "vit. free,"§	10 "		332	6	0			
+ Amino acid mixture	10 "	0	221	6	0	210	17	0
+ Pork kidney powder, dried defatted	2 "		148	6	4			
+ <i>Idem</i>	5 "	108	242	6	0			
+ Factor 3 prep. 3/3722; conc. of α -F3 from pork kidney powder	(1.7 ")¶	270				225	17	0
+ <i>Idem</i>		106				195	17	11
+ Factor 3 prep. 3/3446A; ** conc. of β -F3 from pork kidney powder	(3.4 ")	212						
+ Factor 3 prep. 3/2503; purified α -F3 from pork kidney powder	(3.2 ")	82	169	6	1	210	16	4
Basal	24 mg	51	244	6	1			
+ Selenium, as seleno-cystathionine	10 μ g		131	7	6	157	19	14
+ " " as sodium selenite	10 "	180††	275	4	0	196	17	0
+ " " elemental	200 "	230††	232	7	0	199	18	0
		80††	191	7	2	232	19	0

* Assayed against dietary necrotic liver degeneration in the rat (see *Methods*).

† General Biochemicals, Inc.

‡ Added to diet at beginning of third week.

§ Mostly NaCl.

¶ Loc. cit. 3.

†† Summary of 3 groups of 6 chicks each.

‡‡ Amino acids used in proportions present in casein.

§§ K. Schwarz and C. M. Foltz, unpublished results.

vit. E-free lard 5%, cellulose 3%, glycine 0.5%, L-arginine HCl 0.24%, DL-methionine 0.3%, and adequate amounts of vitamins except E. In both laboratories chicks were started when one day old. They were housed in electrically heated batteries with wire mesh floors. Food and water were given *ad libitum*. Supplements were added by thorough mixing into the diet at the expense of cere-lose.

Results. In Table I a selection of tests carried out at different times at the 2 laboratories is presented. Besides tocopherol, various natural, vit. E-free sources of Factor 3 protected against exudative diathesis. In general, there was good correlation between Factor 3 activity as established in the rat assay, on one hand, and the protective effects obtained in the chick. Dried brewers yeast and vitamin-free casein completely prevented symptoms and promoted good growth when added at 10% levels to the diet. A mixture of amino acids simulating the composition of casein was ineffective. These results are identical to those described for dietary necrotic liver degeneration(10). Fat-extracted pork kidney powder, which is a potent source of Factor 3,[†] gave excellent growth and prevented exudative diathesis at the 2% and 5% level.

The Factor 3 fractions comprise a relatively crude concentrate of α -Factor 3,[§] a concentrate of β -Factor 3 of medium degree of concentration, and a more purified α -Factor 3 fraction which was obtained from pork kidney powder after various steps of purification. These preparations produced degrees of protection in the chick which were predictable from the results obtained in the Factor 3 assay in the rat. The preparation used in the Cornell study (3/3772) reduced exudative diathesis to 25% incidence at the higher level tested. Both preparations tested at Bethesda protected 5 of 6 chicks. One of these gave a good growth response; the other one

was added during the third week of the test.

The identity of Factor 3 with the agent preventing exudative diathesis in the chick was established beyond doubt when it became known that purified preparations of Factor 3 contained organic selenium and that sodium selenite would prevent dietary liver degeneration in rats(3). The 3 forms of selenium were tested simultaneously at both Bethesda and Cornell. In the rat, selenocystathionine fully protected against necrotic liver degeneration when supplied at a level equivalent to 4 μ g of selenium per 100 g of diet. In the chick, this organic selenium compound completely prevented exudative diathesis at levels equivalent to 10 μ g of selenium per 100 g of ration. The same effect was obtained with 10 μ g % of selenium in form of sodium selenite. Elemental selenium, on the other hand, was required at higher dose levels in order to prevent liver necrosis in the rat. In the chick assay, 200 μ g % protected 5 out of 7 animals in the Nat. Inst. of Health study, and all animals at Cornell. All 3 forms of selenium produced a strong growth promoting effect (Fig. 1). Some inconsistencies in growth rates obtained with different preparations are apparent, but the data on hand are insufficient for conclusive interpretations of the discrepancies.

Discussion. In the past, selenium has received a great deal of attention because of its toxicity. The extensive literature on the subject has been reviewed elsewhere(11).



FIG. 1. Effect of selenium on growth and appearance of New Hampshire chicks. Left: Last survivor on unsupplemented basal diet. Right: Chick representing avg of sodium selenite supplemented group.

[§] In acid hydrolysates of kidney powder, at least 2 chemically different but related components with Factor 3 activity have been detected. These have been designated α and β Factor 3. (Schwarz, K., Mason, L. H., and Foltz, C. M., to be published).

The viewpoint prevailing until now could be summed up in a sentence by Trelease and Beath, "as far as we know, selenium is toxic but never beneficial to animals or man" (12). The recent findings—that Factor 3 contains selenium in organically bound form, and that it is replaceable by selenite in μg quantities—indicate that *selenium may be a hitherto unrecognized, essential trace element*. It is seen from the present study that in the chick, as in the rat, selenium affords protection against serious and fatal deficiency syndromes at dose levels which constitute only a very small fraction of the minimal toxic dose.

It is evident from our results that a rather close parallelism exists between the rat and the chick with respect to protective effects of Factor 3 and other selenium compounds under dietary conditions which lead to liver necrosis, or to exudative diathesis, respectively. Both diseases are prevented by crude sources of Factor 3, by Factor 3 concentrates, and by selenium in form of seleno-cystathionine and as sodium selenite. Elemental selenium is also effective.

From the activities of crude sources of Factor 3, and also of Factor 3 concentrates, (Table I), it can be calculated that dose levels effective against exudative diathesis in the chick are approximately 2 to 3 times as high as those required by the rat for protection against liver necrosis. These levels appear to be "physiological" *i.e.*, they correspond to the trace amounts of bound selenium (Factor 3) ingested with normal diets. Comparison of the effective doses of selenium with those of vit. E shows that in the chick selenium in form of selenocystathionine or selenite is 500 to 1000 times as active as tocopherol. This ratio is very similar to that in the rat, which is roughly 500:1 (3). It is obvious that the presence or absence of selenium in the form of Factor 3, or as an inorganic compound, decisively influences the course of vit. E deficiency diseases. In standard rations used for assaying vit. E in the rat, Factor 3 is amply supplied as an impurity in the vitamin free casein (10). The exact relationship between vit. E and Factor 3 remains to be clarified. Preliminary results indicate that the 2 substances act independently

of each other in alternate pathways of intermediary metabolism.[†]

To our knowledge, a biological function has been previously ascribed to selenium only in one instance: Pinsent found that selenite is required for formation of formic dehydrogenase in *E. coli* (13). Results suggestive of a beneficial effect of selenium in the chick were obtained by Poley and others in their study on selenium toxicity (14). However, their experiments do not permit a conclusive interpretation since they were carried out with seleniferous wheat, and not with pure compounds. Subtoxic levels of the element (200 μg %) produced a positive growth response in 5 out of 6 trials. The amounts used in these studies were much in excess of those found effective in the present investigation.

Summary. It is shown that exudative diathesis in the chick, produced by vit. E-free *Torula* yeast diets, is prevented by crude sources and purified fractions of Factor 3. Selenium, recently identified as the integral part of Factor 3, was highly active. Protection was obtained with 10 μg of selenium per 100 g of diet in form of seleno-cystathionine or sodium selenite; 200 μg of elemental selenium also were effective. All 3 forms of the element stimulated growth.

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Received June 20, 1957. P.S.E.B.M., 1957, v95.

Hematocrit Change as Indication of Anaphylactic Shock in the Mouse. (23309)

JOHN D. FULTON, WILLIAM E. HARRIS, AND CHARLES E. CRAFT

Department of Microbiology, School of Aviation Medicine, USAF, Randolph Air Force Base, Texas

Because of their complexity, methods for quantitation of anaphylaxis in large hypersensitive animals(1) are not readily adapted for the mouse. Consequently, the occurrence of anaphylaxis in mice is customarily determined using death as end point. Attempts have been made to estimate sublethal anaphylaxis in mice by observing the appearance of certain gross signs (*i.e.*, cyanosis and convulsions). Because of their subjectivity, these observations are inadequate. Kind(2) found that the rectal temperatures of mice surviving anaphylactic shock were markedly decreased.

In our laboratory we consistently observed that hemoconcentration occurs in mice undergoing anaphylaxis. We observed signs of anaphylaxis similar to those described by Weiser *et al.*(3). Immediately after challenge most of the mice became quiet and huddled in one position. The fur became ruffled and there was mild lacrimation. There soon followed partial paralysis of the limbs and marked cyanosis of the limbs, tail, and snout. Many animals experienced brief but violent convulsions separated by short periods of complete prostration. Nearly all mice which underwent convulsions died within 30 minutes. Nelson *et al.*(4) reported that the hematocrit of pooled mouse blood was markedly increased following fatal anaphylactic shock. The present study was designed to determine whether or not hematocrit change might be a suitable criterion for determination of anaphylaxis in mice.

Methods. Sensitization and challenge. Fol-

lowing randomization, female Swiss-Webster mice (22-27 g) were sensitized with 2 IP injections of 0.25 ml of antigen administered 4 days apart. Twelve days after receipt of the initial sensitizing injection the animals were challenged with an injection in the caudal vein of 0.25 ml of antigen. Two antigen preparations were employed: 1) alum precipitated bovine serum albumin* with *Hemophilus pertussis* vaccine† (APBA + HPV), each 0.25 ml containing 1 mg of bovine serum albumin suspended in 2.5% alum (pH 6.5 to 7.5) with 10^9 to 10^{10} *H. pertussis* vaccine cells; and, 2) the same as above except that *H. pertussis* vaccine was not included. This antigen preparation is referred to as APBA. A single challenge antigen preparation, bovine serum albumin with *H. pertussis* vaccine (BSA + HPV) was used. Each 0.25 ml of this preparation contained 4 mg of bovine albumin and 10^9 to 10^{10} cells *H. pertussis* vaccine. Controls included groups of mice sensitized with one or the other of the 2 sensitizing antigen preparations and challenged with saline, mice injected with saline instead of sensitizing antigen preparation and challenged with BSA + HPV, and groups given saline instead of both sensitization and challenge. *Hematocrit determination:* A modification of the microhematocrit method described by McGovern *et al.*(5) was employed. Four-tenths mm ID microprecipitin capillary

* Bovine serum albumin, 30%, sterile, for use as diluent in Rh testing. Armour Labs.

† Pertussis vaccine (whooping cough bacterin), Wyeth Labs.

tubes† were heparinized by immersing them in a 1% solution of heparin sodium salt,§ then drying at 100°C for 60 minutes. The dried, heparinized tubes were stored in a desiccator until use. To take the blood sample, the tail of an immobilized mouse was severed the minimum distance from the tip required for moderate bleeding. Following collection of blood, one end of the partially filled capillary tube was sealed by flaming, and the open end broken off. The tubes were then centrifuged for 4 minutes at 10,000 rpm in a standard micro-hematocrit centrifuge.|| Hematocrit values were determined by using the standard reading template. Two sequential hematocrits were taken approximately 5 minutes prior to administering the challenge injection. The mean of these 2 hematocrit values is considered to be the prechallenge, normal hematocrit of the animal. For approximately 550 duplicate samples, a standard deviation of ± 1.57 units (approximately 3%) was observed. It can be inferred from this that a hematocrit change of 5% or more is not likely to be due to chance; therefore, a change in hematocrit of 5% or more has been arbitrarily selected as being significant. Approximately 5 minutes after the duplicate prechallenge hematocrits, the challenge dose of antigen or saline was injected. Blood samples were taken at 5, 10, and 15 minute intervals after challenge. In earlier studies blood samples were taken at 2 minutes and at each 5 minute post-challenge interval through the 30 minute period. This was discontinued as it was found that the maximum hematocrit change occurred, if there was a change, within 15 minutes post-challenge. Hematocrit changes were calculated according to the following formula:

$$\% \text{ change} = \frac{\text{Mean post-hematocrit} - \text{Mean pre-hematocrit}}{\text{Mean pre-hematocrit}} \times 100$$

† Capillary tubes, 0.4 mm ID, 90 mm long. Schiefelin & Co., N. Y.

§ Liquaemen, Roche-Organon, Nutley, N. J.

|| International hematocrit centrifuge, Interntl. Equipment Co., Boston, Mass.

Results. Sensitivity and specificity of hematocrit rise during anaphylaxis. Test animals were treated as follows:

Sensitization	Challenge	Approx. No. mice
APBA + HPV	BSA + HPV	150
APBA	" "	150

Control animals were treated as follows:

Sensitization	Challenge	Approx. No. mice
APBA + HPV	Saline	100
APBA	" "	100
Saline	BSA + HPV	150
"	Saline	100

Seventy-five % (221/295) of test animals showed a hematocrit increase of 5% or more, and 41% (121/295) showed a hematocrit increase of 30% or more (Fig. 1). In the test group, there were 47% (139/295) deaths within 24 hours after challenge (Fig. 2). Of the 139 test animals that died, 133 showed a hematocrit rise of 5% or more. Thirty % (88/295) of test animals had a hematocrit increase of 5% or more but did not die. In the control group, 3% (12/446) had a hematocrit rise of 5% or more (Fig. 1). In this group 3% (13/446) died (Fig. 2), but only one of the 13 was among the 12 control mice showing a 5% hematocrit rise. The 12 remaining control animals that died showed a decreased hematocrit (Fig. 2).

Comparison of hematocrit change and mortality rate as methods for measuring anaphylaxis. In this experiment, 2 groups of approximately 45 animals each were employed for each of 2 sensitizing procedures,

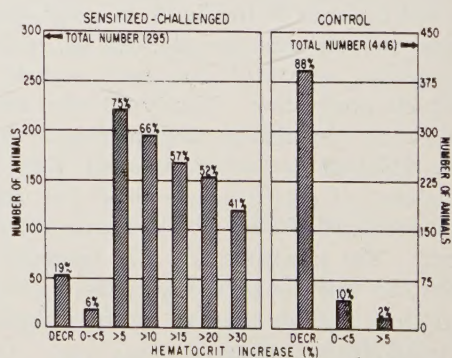


FIG. 1. Hematocrit response of sensitized-challenged animals vs control animals.

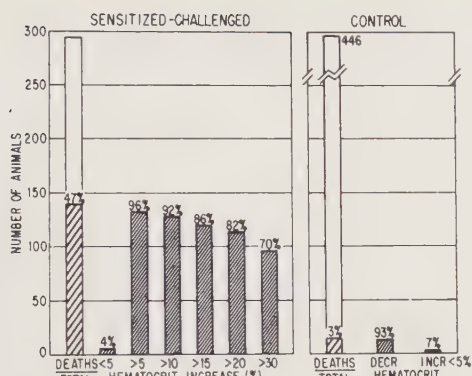


FIG. 2. Relationship between anaphylactic death and hematocrit rise.

viz., sensitized with APBA + HPV, and sensitized with APBA (Fig. 3). In both procedures, the same challenge antigen (BSA + HPV) was employed. Control groups to complete the factorial design (as in the preceding section) were included. One group of mice was sensitized and challenged, and hematocrits taken as described previously. The second group of mice was sensitized and challenged, was observed, and deaths within the 24-hour post-challenge period recorded. The animals in this group were immobilized for the same period of time as was the "hematocrit group."

Comparison of results obtained in this experiment indicates that the incidence of hematocrit rises of more than 5% was higher than the incidence of deaths ($P < .001$) (Fig. 3). It had previously been observed (unpublished results) that the 2 hypersensitizing technics described above produced significantly different death rates following challenge; this was confirmed in the present study ($P < .05$) (Fig. 3). The 2 different sensitizing procedures also resulted in differences in the number of animals showing hematocrit increases of 5% or more ($P < .01$) (Fig. 3).

Discussion. It has been shown that, with the technics employed, an increase in hematocrit regularly accompanies anaphylaxis in mice. In a group of animals of which 47% died in anaphylaxis, 75% showed a hematocrit increase of 5% or more. This would indicate that increased hematocrit occurs both in mice that subsequently die in anaphylaxis and in those which survive. Control animals

TREATMENT*	HEMATOCRIT INCREASE >.05	MORTALITY RATE
APBA+HPV BSA+HPV	96% (45/47) $\chi^2 = 18.1$ $p < .001$	58% (24/41)
APBA BSA+HPV	71% (32/45) $\chi^2 = 12.9$ $p < .001$	33% (15/45)

*SENSITIZING ANTIGEN
CHALLENGE ANTIGEN

FIG. 3. Comparison of hematocrit change and mortality counts as methods for determining degree of anaphylactic shock.

consistently showed a decrease in hematocrit. When the incidence of mortality and hematocrit change in different groups of mice is compared, hematocrit increases of 5% or more were found to occur more frequently than death in groups of anaphylactic animals. Differences were observed in the incidence of anaphylactic responders produced after challenge of animals made hypersensitive by 2 different sensitizing procedures when either death or hematocrit increase was used as an end point. It appears, therefore, that hematocrit increase may be a more sensitive index of anaphylaxis than death.

Summary. An increase in hematocrit regularly accompanies anaphylaxis in mice. The incidence of hematocrit increase is higher than the incidence of death in groups of anaphylactic mice. It is suggested that hematocrit change is a useful index of anaphylaxis in mice.

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Received January 21, 1957. P.S.E.B.M., 1957, v95.

Renal Localization, Excretion and Toxicity of Mercuric Chloride in Normal and "Nephrotic" Rats.* (23310)

A. SURTSHIN AND ALLEN G. PARELMAN (Introduced by A. S. Gilson, Jr.)

Department of Physiology, Washington University School of Medicine, Saint Louis

During studies on the toxicity of mercuric chloride we have tested rats with an experimental nephrotic syndrome. Rats given a course of injections of an aminonucleoside related to "puromycin" develop an increase in proteinuria, hypoproteinemia, edema and ascites. The clinical state and renal lesions have been described as resembling somewhat those present in the nephrotic syndrome in children (1). We have found that such "nephrotic" rats survive intravenous doses of mercuric chloride uniformly fatal to normal animals, although the mercury contents of the kidneys, collected urines and collected feces, as revealed by analysis 24 hours after injection, do not differ significantly between the normal rats and those with the experimental nephrotic syndrome.

Method. Sprague-Dawley males offered tap water and Purina Laboratory Chow were used. To compare the toxic effects of mercuric chloride in normal and "nephrotic" rats, 20 animals weighing between 142 and 200 g were injected subcutaneously once daily for 8 days with 0.003 ml per g of body weight of a 0.5% aqueous solution of 6-dimethylamino purine, 3-amino-d-ribose.[†] Plasma protein concentrations were determined (6) before the initial and 48 hours after the final injection. Within a few hours after the second blood sampling, 15 of the 20 rats were given mercuric chloride, 3 mg per kilo of (edematous) body weight, via the femoral vein. All 20 rats were then observed for 4 weeks or more. Survival rate of the mercuric chloride injected animals was compared with that of 12 mercuric chloride injected previously untreated normal rats on the same offered diet. Six additional rats weighing 165 to 195 g were given

12 daily subcutaneous injections of the aminonucleoside as above. Plasma protein concentrations were determined before the first and 48 hours after the final injection. Within a few hours after the second blood sampling the rats were given $\text{Hg}^{203}\text{Cl}_2$ intravenously, 3 mg per kg of (edematous) body weight and put into individual collection cages. Twenty-four hours after the injection they were weighed and killed. The kidneys were excised, weighed rapidly and placed in a tube for counting. Also assayed were aliquots of the diluted urine after washing of the cage, the total feces, the pledget used at the point of venepuncture and a block of tissue excised at the injection site. Aliquots of the diluted urine were analyzed also for protein (8). The remainder of the carcass, including all the fluid contents, was dried to constant weight in an oven between 80° and 90°C. The total solids content of 3 normal rats of the same weight group was also determined; using the mean percentage of body solids found in these 3, the "ideal" (non-edematous) body weights at the time of their sacrifice were calculated for the edematous rats. Six normal rats of appropriate weight also given intravenous radiomercuric chloride and similarly treated were used as controls. The $\text{Hg}^{203}\text{Cl}_2$ had been prepared with HCl from Hg^{203}O and was of low activity, giving in our apparatus from 35,300 to 25,400 counts per mg of $\text{Hg}^{203}\text{Cl}_2$ per minute. Counting was done with a Model DS 3 Nuclear-Chicago well type scintillation counter and a Model 192 Nuclear-Chicago Ultrascaler. Counts were to at least 10,000 for backgrounds, 40,000 for standards, 10,000 for kidneys, 1,000 for urines, 4,000 for feces. The mean statistical errors of determination for urine and feces are, therefore, because of the low level of radiation and the low number of total counts, relatively high, being 10.5% for urine and 2.6% for feces. For kidneys the error is

* Aided by grants from Amer. Heart Ass'n and NIH, U.S.P.H.S.

[†] We are indebted to Dr. Stanton M. Hardy of Lederle Laboratories, Division Amer. Cyanamid Co., Pearl River, N. Y. for this material.

only 1%. No significant amounts of Hg^{203} were detected in the pledgets nor was leakage at the site indicated by the activity of any of the site areas excised.

Results. Toxicity of mercuric chloride in normal rats and rats with nephrotic syndrome. The mean plasma protein concentration of the 15 rats receiving stable HgCl_2 was 6.04 ± 0.76 g % before initial injection of the aminonucleoside and 4.60 ± 0.37 g % at the time of HgCl_2 administration. That of the 5 animals which received the aminonucleoside, but not mercuric chloride, was correspondingly 5.68 ± 0.78 g % and 4.59 ± 0.40 g %. All 20 appeared to have ascites on the tenth day. Of the 15 rats given HgCl_2 , 14 were still alive and no longer ascitic 4 weeks after the injection. They had gained weight and were in apparent good health when killed. All 5 not given mercuric chloride were also in apparent good health at this time. One rat died 48 hours after mercuric chloride administration, having had the highest plasma protein (5.4 g %) of the group. This survival rate contrasts with that of normal untreated rats given 3 mg per kg HgCl_2 intravenously in which of 12 injected all died within 6 days, with diarrhea and hematuria.

Excretion and renal localization of $\text{Hg}^{203}\text{Cl}_2$ by normal rats and rats with nephrotic syndrome. Three uninjected normal rats were found to have solids contents of 31.0, 31.4 and 30.5% of the body weights. Therefore, the factor of 0.31 was used to convert the desiccated carcass weights of the "nephrotic" and normal animals given $\text{Hg}^{203}\text{Cl}_2$ to "ideal" weights at the time of sacrifice (desiccated weight divided by 0.31).

The data are summarized in Table I. The mean weights of the desiccated carcasses (actually, carcasses minus kidneys and injection sites) for the "nephrotic" and normal groups, respectively, were 44.9 and 42.2 g. These are convertible to mean "ideal" weights of 144.5 (range 127-160) and 136.2 (range 126-148) g at time of sacrifice for the "nephrotic" and normal groups, respectively, with mean excesses in body water content of 42.7 and 15.2 g, as compared with uninjected controls.

After radiomercuric chloride injection the normal group had diarrhea and hematuria,

manifestations of acute mercury poisoning.

Discussion. Holman and Donnelly(3) and Martin and Reid(7) found that dogs made hypoproteinemic by plasmapheresis survived intravenous administration of 3 mg per kilo HgCl_2 , a dose fatal to normal dogs. On the other hand, Havill, Lichty and Whipple(2) found that frequent injection of hemoglobin into dogs increased their tolerance for mercuric chloride, and Lippman and his associates reported that the toxic effects of mersalyl-theophylline, meralluride sodium (Mercuhydrin)[®] or mercuric chloride on the kidney in rats were reduced by the prior administration intraperitoneally of human or bovine serum albumin(4,5). The latter workers ascribed the protection to presence of albuminuria in their animals and suggested that both blockage of tubular reabsorption of mercury and an increase in urinary excretion of mercury due to binding by urinary protein might be the mechanisms operating.

Our rats with both hypoproteinemia and an increase in proteinuria clearly show an increase in resistance to the toxic effects of mercuric chloride. The dosage, 3 mg per kg, is higher than this when expressed in terms of the "ideal" weights. We have not analyzed the ascitic fluid for mercury, but ascitic fluid in human patients with hepatic cirrhosis given Mercuhydrin[®] contained mercury in only low concentration(9). We found no significant diversion of mercury from the kidney in the presence of the high excess of body water.

Our data show no significant differences between the "nephrotic" and normal groups with respect to the percentage of the administered Hg^{203} present at 24 hours in the kidneys ($P > 0.1$), in the collected urine ($P > 0.05$), and in the collected feces ($P > 0.1$). The cause for the protection against mercuric chloride is not apparent. Failure to correlate such protection with amounts of mercury found in the kidneys was found also in rats in which an increase in resistance to the toxic effects of mercuric chloride was induced by feeding a diet of sucrose and vitamins(15). Although Lippman *et al.*, with rats protected by pretreatment with intraperitoneal serum albumin, interpreted their data as showing a reduction in the renal localization of Hg^{203}

TABLE I. Excretion and Renal Localization of $\text{Hg}^{203}\text{Cl}_2$ in Normal Rats and Rats with Experimental Nephrotic Syndrome, 24 Hr after Intravenous Injection of 3 mg/kg $\text{Hg}^{203}\text{Cl}_2$.

Initial wt, g	Wt at inj., g	Wt at sac- rifice, g	Kidney wt, g	Dry wt, g	Body solids, %	Dosage* Hg ²⁰³ Cl ₂ , mg/kg	Hg ²⁰³ dose distribution (%)†			Plasma protein (g %)		Urinary protein, mg/24 hr
							Kidney	Urine	Feces	Initial	Final	
Rats with nephrotic syndrome												
186	216	204.1	3.07	45.3	22.54	4.43	15.9	14.7	5.5	5.37	4.39	118
166	194	158.8	2.76	39.5	25.32	4.57	17.4	17.7	1.2	7.00	4.91	302
195	190	167.1	2.23	41.6	25.23	4.25	20.2	10.5	2.7	6.27	4.15	97
198	220	201.3	2.93	46.1	23.24	4.44	19.2	21.8	4.2	5.71	<4.00	258
195	218	206.3	2.43	47.3	23.20	4.29	10.7	13.9	5.4	6.48	4.01	259
199	207	203.7	3.12	49.5	24.68	3.89	16.4	19.2	4.2	6.24	4.04	208
	Mean		2.76	44.9	24.04	4.31	16.6	16.3	3.9	6.18	4.25	207
	S.D.				±1.18	±.24	±3.3	±4.1	±1.7	±.57	±.34	±83
Normal rats												
155	161.7	141	1.41	44.0	27.45	4.33	17.2	7.4	1.6			18
140	138.3	1.29	1.29	38.9	28.39	4.70	18.8	9.7	6.5			35
174	168.0	1.70	1.70	45.9	27.60	4.63	21.0	9.2	4.5			19
149	134.4	1.33	1.33	39.2	29.45	4.78	16.3	16.2	2.0			42
168	164.8	1.77	1.77	43.8	26.87	3.89	18.8	15.4	5.7			20
164	150.5	1.75	1.75	41.5	27.89	4.53	19.5	5.5	1.2			37
	Mean		1.54	42.2	27.94	4.48	18.6	10.8	3.6			28.5
	S.D.				±.89	±.33	±1.9	±4.1	±2.3			±10.7

* mg $\text{Hg}^{203}\text{Cl}_2$ /kg of "ideal" (nonedematous) body wt at sacrifice (see text).

† % of inj. dose found in kidney, urine and feces.

and an increase in its excretion(5), we have interpreted their data differently and have ourselves found a renal mercury content slightly greater in albumin-injected rats than in saline-injected controls when both sets were sacrificed 24 hours after mercuric chloride injection(11).

Summary. Rats given 8 or 12 daily injections of 6-dimethylamino purine, 3-amino-d-ribose, develop increase in proteinuria, hypoproteinemia, edema and ascites. Such rats survive an intravenous dose of mercuric chloride lethal to normal rats. The mercury contents of kidneys, collected urine and collected feces, 24 hours after intravenous injection of $\text{Hg}^{203}\text{Cl}_2$, are not significantly different in rats with the experimental nephrotic syndrome from those in normal rats given the same dose.

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Effect of Hypothermia on Ventricular Fibrillary Threshold. (23311)

BENJAMIN G. COVINO* AND WILLIAM R. BEAVERS (Introduced by K. Rodahl)

Arctic Aeromedical Laboratory APO 731, Seattle, Wash.

Cardiac arrhythmias occur with alarming frequency under conditions of experimental hypothermia(1,2). A hyperirritable ventricle brought about by the hypothermic process is believed responsible for these cardiac irregularities, of which ventricular fibrillation is the most common. Direct measurements of ventricular threshold thus far have failed to reveal any difference between excitability of the normo- and hypothermic heart (3,4). However, no information is available as yet on the actual threshold for ventricular fibrillation at various body temperatures. Thus quantitative data on ventricular fibrillary threshold during progressive hypothermia appear necessary to confirm or refute the hypothesis of a hyperirritable hypothermic heart. During our study, an attempt was made to measure ventricular fibrillary thresholds by determining the minimum electrical

stimulus needed to induce a fibrillary state in dogs subjected to immersion hypothermia. In addition, the effect of pH and prolonged cold exposure on ventricular fibrillary threshold was evaluated. Previous studies have revealed that cold acclimatization(5) and maintenance of normal pH during general body cooling(6) significantly reduce the incidence of hypothermic ventricular fibrillation. Thus, ventricular fibrillary thresholds were measured in cold acclimatized normocapneic dogs during hypothermia to determine if these procedures exert their beneficial effect by direct action on the myocardium.

Method. Eight apparently healthy dogs, ranging 13 kg to 25 kg, were anesthetized with intravenous pentobarbital sodium (30 mg/kg). The left common carotid artery was exposed and cannulated for measurement of mean arterial blood pressure via a mercury manometer. Samples of venous blood were obtained from the left jugular vein at rectal

* Present address: Department of Pharmacology, Tufts Medical School, Boston, Mass.

temperatures of 38°, 30°, 22°C for determination of serum pH in a Cambridge pH meter equipped with a water jacket. This made possible the measurement of pH at the actual temperature of the blood. A maximum time interval of 1 minute elapsed between blood sampling and pH measurement to insure a minimum change in blood temperature. Standard limb leads were attached and electrocardiographic tracings (lead 2) were obtained on a Sanborn poly-viso cardiette. All animals were intubated with endotracheal tube and artificial respiration (push-pull) with room air was instituted as rectal temperature fell below 30°C. Rectal temperatures in °C were recorded continuously on a Leeds-Northrup iron-constantan potentiometer. Three dogs were rendered hypothermic following 4 weeks of continuous exposure to an environmental temperature of 0 to -40°C. This was done to evaluate the influence of cold acclimatization on the ventricular fibrillary threshold. Direct quantitative measurements of the ventricular threshold were made as described previously(7,8). During our study, however, both the driving and testing electrode were attached to the left ventricle. Two Grass square wave stimulators connected in tandem were used to drive and test the threshold of the ventricle. Anodal stimuli, varying from 0-15 mamp. and duration from 3-10 msec., were delivered at 10 msec. intervals throughout the cardiac cycle to determine the excitability recovery curve of the ventricle. The minimum stimulus required to produce a single extrasystolic beat was taken as a measure of the basic ventricular threshold. The ventricular fibrillary threshold was determined in a similar fashion by delivering at 10 msec. intervals throughout the cardiac cycle anodal stimuli of 0-15 mamps. and 3-10 msec. duration. It was found, however, that ventricular fibrillation could only be produced when impulses were applied to the ventricle immediately prior to diastole. Thus, the fibrillary threshold represents the minimum stimulus, in terms of strength of current and duration, which can produce ventricular fibrillation when applied during the prediastolic period of the cardiac cycle. Stimulation studies were carried out initially at

normal body temperature, following which the dogs were immersed in a recirculating cold water bath of 3.5°C. The studies were then repeated at rectal temperatures of 30°C and 22°C.

Results. The basic threshold of the normothermic ventricle was determined in all cases prior to measuring the fibrillary threshold. The excitability recovery curve was essentially similar to that reported previously by Hoffman *et al.*(9). A period of absolute refractoriness, during which a maximum stimulus of 15 mamp. was ineffective, occurred immediately following ventricular depolarization. This interval was followed by a relative refractory period during which the ventricular threshold fell sharply within 20-70 msec. from a level of >15 mamp. to 1.2-2.6 mamp. A period of relative hyperexcitability immediately prior to diastole was observed and corresponded to the major dip of Hoffman *et al.*(9) and the vulnerable period of Wiggers and Wegria(10). No minor dip early in the cardiac cycle was observed. This may be due to the fact that our maximum strength of stimulus was 15 mamp. and anodal in nature.

Attempts to fibrillate a normothermic ventricle by gradually increasing the strength of stimulus to a maximum of 15 mamp., and the duration of stimulus from 3 to 10 msec. were uniformly unsuccessful. In 2 instances, a stimulus of 10 mamp. applied immediately prior to diastole did produce multiple ectopic beats but in no case was ventricular fibrillation initiated. Impulses delivered during the relative refractory period or diastole never induced more than a single extrasystolic beat.

At a rectal temperature of 30°C, no change in ventricular excitability recovery curve was observed. The 3 phases of the cardiac cycle, absolute refractory period, relative refractory period, and diastole, were slightly prolonged due to the decrease in heart rate at this temperature. However, the threshold levels during these various periods were similar to those observed in the normothermic heart. Again ventricular fibrillation could not be produced by application of stimuli of maximum strength to the ventricle at any point in the cardiac cycle. As rectal temperature fell to 22°C

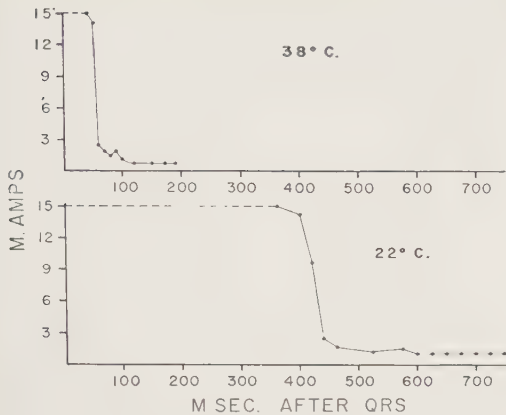


FIG. 1. Comparison of excitability recovery curve at rectal temperatures of 38°C and 22°C.

and the mean serum pH to 7.18, the average heart rate decreased to 42 beats per minute. The results of this bradycardia was a prolongation of the cardiac cycle. Yet no significant difference obtained between the basic ventricular threshold at 38°C and 22°C (Fig. 1). However, the ventricular fibrillary threshold was greatly diminished. At this temperature a stimulus of 2.5-5.2 mamp. was sufficient to initiate ventricular fibrillation in all dogs. Table I reveals the actual changes in diastolic threshold and ventricular fibrillary threshold which occurred during cooling in each of the 5 experimental animals. The average diastolic threshold remained quite constant, 1.32 mamp. at 38°C and 1.42 mamp. at 22°C, yet, the average fibrillary threshold decreased from >15.0 mamp. at 38°C to 3.44 mamp. at 22°C. Thus, the basic ventricular threshold is not affected by the hypothermic episode, while at least a 5 fold decrease in mean fibrillary threshold occurred

TABLE I. Comparison of Diastolic and Ventricular Fibrillary Thresholds at Rectal Temperatures of 38°C and 22°C, in 5 Dogs.

38°C		22°C	
Diastolic threshold (mamp.)	V.F. threshold (mamp.)	Diastolic threshold	V.F. threshold
.7	>15	1.1	3.5
.8	"	1.4	2.5
1.9	"	1.9	5.2
2.05	"	1.8	3.4
1.2	"	1.9	2.6
Avg 1.32	"	1.42	3.44

TABLE II. Comparison of Diastolic and Ventricular Fibrillary Thresholds at Rectal Temperatures of 38°C and 22°C in 3 Cold Acclimatized, Normocapneic Dogs.

38°C		22°C	
Diastolic threshold (mamp.)	V.F. threshold (mamp.)	Diastolic threshold	V.F. threshold
2.0	>15	2.0	3.5
.9	"	.7	4.0
.8	"	1.3	2.5
Avg 1.23	"	1.33	3.33

when rectal temperature was lowered to 22°C.

Effect of pH and cold acclimatization. Three dogs were exposed to an environmental temperature of 0 to -40°C for 4 weeks and then rendered hypothermic. The serum pH was maintained between 7.30 and 7.50 in all dogs via controlled artificial respiration. Table II shows that the diastolic threshold again was unaffected by the reduction in body temperature. The ventricular fibrillary threshold, however, fell from >15 mamp. at 38°C to a mean of 3.33 mamp. at 22°C. No difference in fibrillary threshold existed at 22°C between acclimatized, normocapneic dogs and non-acclimatized hypercapneic dogs. Thus, neither cold acclimatization nor maintenance of a normal pH during hypothermia was capable of preventing the fall in ventricular fibrillary threshold produced by the general reduction of body temperature.

Discussion. Our data demonstrate quite conclusively that the nembutalized hypothermic dog is highly susceptible to cardiac arrhythmias and particularly ventricular fibrillation, and affords the first direct evidence that a decrease in ventricular fibrillary threshold actually does occur during hypothermia. Although the fibrillary threshold was significantly diminished during general body cooling, no difference could be demonstrated between the basic ventricular threshold of the normo- and hypothermic heart. The latter also has been reported by Brooks *et al.*(3) and Hegnauer *et al.*(4). These findings indicate the fallacy of attempting to evaluate the efficacy of various antifibrillary procedures by direct determination of the diastolic ventricular threshold alone. It is apparent from our results that the latter may

not serve as an accurate index of the hypothermic heart's susceptibility to ventricular fibrillation. This situation, however, may be peculiar to hypothermia since Hoffman *et al.* (9) have reported that both the diastolic threshold and the fibrillary threshold of the normothermic ventricle are altered in a similar fashion following injection of epinephrine.

The failure to observe a change in diastolic threshold suggests that cellular processes responsible for the resting excitability of ventricular musculature are effected minimally by the degree of hypothermia utilized in this study. The ventricular fibrillary threshold, however, can be altered by factors other than changes in tissue excitability. Disturbances in cardiac conduction, refractoriness, and pacemaker activity are known to increase the vulnerability of the heart to fibrillation(3). At body temperatures below 25°C ventricular conduction is depressed in a heterogeneous fashion(12) and pacemaker activity is 20% of normal(2). Thus, conditions existing within the hypothermic ventricle are ideal for initiation of disorganized activity. In such circumstances an extrinsic stimulus which is not effective at normal temperature could induce ventricular fibrillation at low temperatures due to the presence of favorable intrinsic conditions. The normal diastolic threshold and reduced fibrillary threshold observed in hypothermia tend to support this thesis. These altered intrinsic factors may also be responsible for spontaneous hypothermic ventricular fibrillation. Potential ectopic foci may discharge more readily in the presence of a depressed pacemaker. In addition, the heterogeneous ventricular conduction pattern would allow the reentry of ectopic impulses and so produce repetitive firing and fibrillation.

It is interesting to note that 2 procedures, which are capable of decreasing the incidence of spontaneous fibrillation during hypothermia were ineffective in checking the sharp reduction of ventricular fibrillary threshold.

Maintenance of a normal serum pH(5) and prolonged cold exposure(6) both have been shown to influence in a beneficial manner the terminal cardiac event in hypothermic dogs. Thus, the results obtained during our study indicate that the protective effect of these procedures is not due to a direct action on the ventricular myocardium.

Summary. Direct quantitative measurements of both basic ventricular threshold and ventricular fibrillary threshold were made in dogs prior to and during progressive immersion hypothermia. No change in basic ventricular threshold was observed as rectal temperature fell from 38°C to 22°C. At the same time, ventricular fibrillary threshold showed a 5-fold decrease. Neither maintenance of a normal serum pH during cooling nor prolonged cold exposure prior to the hypothermic episode altered the decrease in ventricular fibrillary threshold.

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Received March 22, 1957. P.S.E.B.M., 1957, v95.

Diuretic Effects of Some Mercurated Substituted N-n-propylurea Derivatives. (23312)

HARRY ROSEN, ALBERT BLUMENTHAL, MARY H. NEAD, RICHARD TISLOW AND
JOSEPH SEIFTER

Wyeth Institute for Medical Research, Radnor, Pa.

Since the observation by Vogl(1), that organic mercurials had a diuretic effect, their use as parenteral diuretics has become well established. Recently, considerable effort has been expended toward the development of orally active organomercurials and in this paper we are reporting studies with 6 such mercurial compounds tested for their diuretic effects when administered by the oral route in dogs.

Methods. Various test animals and numerous methods have been used to determine diuretic and antidiuretic properties of drugs(2-6) and the dog appears to have become the test object of choice for evaluating mercurial diuretics(7-10). The animals used in this study were healthy mongrel dogs of both sexes maintained on a standard diet in air-conditioned quarters. They were fasted for 18 hours prior to use, but permitted water *ad lib*. On the morning of the experiment, each dog was given an oral load of 0.9% saline, 25 ml per kg, and one hour later was anesthetized with sodium pentobarbital, 40 mg/kg i.p. The level of anesthesia was kept constant with supplementary doses, as needed. Each dog was catheterized as soon as anesthetized and the bladder emptied by light compression. The catheter remained in the bladder until conclusion of the experiment. Female urethral catheters (12 F.) were used for the female animals and graduated ureteral catheters with a whistle tip (7 or 9 F.) for the male dogs. Collection of urine for experimental purposes was started 2 hours after the saline load and was measured at 15 minute intervals for a total of 5 hours. Each dog was tested twice, thereby serving as his own control. The control experiment was made first and 7 days later, the test experiment was made with the drug under investigation or with a second control. In this way, we have accumulated data which show the urine output that can be expected from the second

TABLE I. Compounds Tested.*
 $\text{NH}_2\text{CONHCH}_2\text{CHRCH}_2\text{HgCl}$.

Wyeth No.	R
1031	$\text{N}(\text{CH}_3)_2$
864	$\text{N}(\text{CH}_2\text{CH}_3)_2$
1032	$\text{N}(\text{CH}_2\text{CH}_2\text{CH}_3)_2$
935	
774	
775	

* The clinical diuretic efficacy of Wy-864 and 935 was reported by David Baden, Thomas Lau, Richard Borreson and Don W. Chapman, *Am. Soc. Pharm. & Exp. Therap.*, French Lick, Ind., Nov. 8-10, 1956.

test without a diuretic. A 2-fold increase in urine output per kg per 5 hours over and above the control output indicated diuresis. Test compounds were administered orally in capsules 60 minutes after the saline load and just prior to anesthesia. Six mercurated substituted-N-n-propylurea derivatives synthesized by Dr. Gerhard Wendt, and listed in Table I by code numbers and corresponding chemical configurations, were all tested at doses equivalent to 2, 4 and 8 mg mercury/kg dog.

Results. One hundred and thirty-seven dogs of both sexes ranging in weight from 4.0 to 16.2 kg were used in this study. The average weight of all dogs for the initial control run was $10.36 \text{ kg} \pm 2.01$. For the test run, 7 days later, the average weight was $10.16 \text{ kg} \pm 2.19$.

Table II shows hourly and total 5 hour urine output for all dogs during the initial control determinations. The averages for males (72) and females (65) as well as combined averages are shown. As time elapsed, hourly output increased for both sexes and average total urine volume was somewhat greater in females.

TABLE II. Control Urine Values in ml/kg.

Collection period	♂ (72)		♀ (65)		Combined (137)	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
1st hr	.6	.45	.8	.40	.7	.43
2nd	.7	.50	.9	.56	.8	.54
3rd	.8	.48	1.0	.63	.9	.57
4th	.9	.55	1.2	.69	1.1	.64
5th	1.0	.56	1.3	.66	1.1	.62
5 hours	4.0	2.08	5.2	2.44	4.6	2.32

During these experiments we accumulated data on 29 dogs (14 males and 15 females) used as double controls; an initial control run and 7 days later another control run without a test drug. Diuretic values were obtained by subtracting the first 5 hour volume of urine in ml per kg from the second 5 hour volume of urine and then dividing the initial volume into this difference. The mean values for males were $0.002 \pm$ a standard deviation of 0.3440 and for females— 0.123 ± 0.3636 . The differences are negligible and an analysis of variance(11) showed no significant difference between males and females or within the groups themselves ($P = > 0.2$). Therefore the data on males and females were combined. A *t* test was made comparing this combined mean diuretic value with an ex-

pected mean of zero. In this case $P = > 0.3$, showing that both control runs, 7 days apart, are equivalent in urine output.

Table III is a summation of the results obtained with each compound. All dogs recorded as positive gave a diuretic value of 2 or greater. Of the 108 dogs included in this table, only 6 as indicated, resulted in questionable diuresis as determined by our standards. The diuretic values for these were between 1 and 1.4, which we considered as a mild diuresis and for our purposes were listed as negative.

Summary. 1) A method for testing mercurial diuretics by the oral route of administration in dogs has been presented. 2) Six mercurated substituted-N-n-propylurea derivatives have been tested by this method and found to be active diuretics.

TABLE III. Summary of Diuretic Results.
No. dogs positive/No. dogs used.

Wyeth No.	Dose (mg mercury/kg dog)		
	8	4	2
774	4/4	0/ 4	2/4
775	2/4*	3/ 4	2/4*
864	3/4	18/24*	2/4*
935	4/4	18/24*	3/4
1031	2/2	4/ 4	3/4
1032	1/2	2/ 4*	0/4

* One dog, considered as negative, gave a diuretic value between 1.0 and 1.4.

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Received April 11, 1957. P.S.E.B.M., 1957, v95.

Propagation of Poliovirus in Chick Embryo Cell Cultures. I. Cultivation of 3 Virus Types. (23313)

WOLCOTT B. DUNHAM AND FRANCES M. EWING

Medical Research Laboratory, Veterans Administration Medical Teaching Group Hospital, Memphis, Tenn.

Cells grown in culture are in certain instances susceptible to infection by a particular virus even though the tissue from which the cells were derived is not capable of being infected in the living animal(1,2). The results of the present study reveal another instance of this biological difference between cells *in vitro* and *in vivo*. We have been unsuccessful in numerous attempts to establish strains of Type 2 poliovirus in the chorioallantoic membrane (CAM) of chick embryos. However, strains of cells derived from this membrane and grown *in vitro* were readily infected with not only Type 2 poliovirus, but also representative strains of Types 1 and 3. The virus had a cytopathogenic effect and multiplication occurred.

Materials and methods. Cell cultures. CAMs from 10-day chick embryos were cut into 1-2 mm pieces, washed in Hanks' solution (H) and mixed with chick embryo extract (CEE)*, 0.1 ml per membrane. The CEE had been centrifuged during preparation at 4000 rpm for 30 minutes and frozen twice before use. About 0.1 ml suspended tissue and 1 ml frozen and thawed chicken plasma (CP)* were introduced into each 4 x 13 cm culture bottle. When clotting had occurred, 10 ml of 40% centrifuged human serum and 60% H (HS₄₀H₆₀)[†] was added. This medium was kept at 4°C for at least 2 weeks before use. The cultures were incubated at 36°C. Every 2-3 days when the medium had become acid, 5 ml medium was removed and replaced with 5 ml HS₄₀H₆₀. The first transfer was made when the sheet of cells began to break up due to partial release of fibrin. The medium was removed and to each bottle was added 10 ml 1% Bacto trypsin in H. After

2 hours at 36°C the contents were centrifuged and the sediment washed twice with H. The sediment was resuspended in H, 2 ml per original bottle. The tissue fragments were allowed to settle for 10 minutes when the supernate, containing chiefly individual cells, was removed and centrifuged. The sediment was resuspended in HS₄₀H₆₀, approximately 2 ml per bottle depending on the concentration of cells. This was transferred in 1 ml amounts to Leighton tubes containing fibrin clots on the flat areas. While establishing the strains, cultivation and transfer in tubes were the same as with the bottles except that volumes were 1/10 as large. These CAM cells were used for virus studies after 5 or more transfers when the cultures were free of tissue fragments and infection produced uniform cytopathic changes. As the cells grow, the fibrin clot becomes somewhat loosened from the glass. To prepare cultures for virus inoculation, the clot was teased off leaving cells on the glass. These were fed and inoculated when sufficient growth had occurred. The fibrin, containing most of the cells, was digested with trypsin and the cells transferred to fresh fibrin clots. *Virus studies.* The poliovirus strains employed were Mahoney, Type 1; Y-SK, Type 2; and Saukett, Type 3. These were obtained through the courtesy of Dr. Jerome T. Syvertson, University of Minnesota. Stock suspensions consisting of centrifuged maintenance solution from infected HeLa cell cultures were stored at -17°C until used. CAM cell cultures were rinsed 3 times with H before inoculation. To each culture tube were then added 0.9 ml of a mixture of 10% chicken serum and 90% Scherer's maintenance solution (CHS₁₀-MS₉₀)* and 0.1 ml virus suspension consisting of stock or fluid from a previous passage of virus in CAM cells. Virus titrations were performed by similar inoculations of cultures of HeLa or CAM cells using dilutions of virus

* Obtained from Microbiological Associates, Washington, D.C.

† Obtained from the Carver Foundation, Tuskegee, Ala.

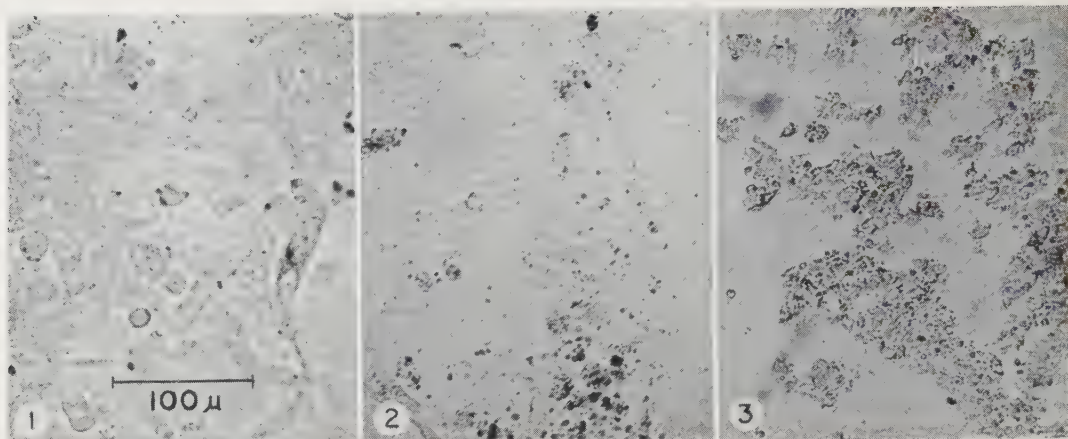


FIG. 1. Control CAM cells. Phase contrast.

FIG. 2. CAM cells showing partial degeneration 2 days after inoculation with poliovirus, Type 2.

FIG. 3. Similar cells showing degeneration at 4 days.

suspensions in CHS₁₀MS₉₀. Infection was assumed if there was +++ destruction of cells and no degeneration in the uninoculated controls. Tissue culture infectious doses were calculated by the method of Reed and Muench(3). Identification of the cytopathogenic agents was accomplished by neutralization tests. Virus suspensions, diluted 1:10, were mixed with equal volumes of polio type-specific monkey immune serum, 1:50, for 1 hour at 27°C and then used in 1.0 ml amounts to inoculate HeLa cells.

Results. CAM cells. Strains of CAM cells were established 3 times. All gave apparently identical results. More than 30 transfers have been made of one strain with no observed change in susceptibility to infection with poliovirus. Early transfers of CAM cells contain many spindle-shaped and round cells that are markedly granular. After some 5 transfers the proportion of spindle-shaped cells diminishes and polygonal cells become prominent, growing in small sheets (Fig. 1). The cytoplasm of these cells is almost clear. Occasional cells have narrow processes that are frequently 3 times as long as the diagonal of the angular cells. A few round cells persist for at least 30 transfers. Time-lapse cinephotomicrographs have not revealed movement or changes in shape of the round cells. Some of the polygonal cells, however, show active pinocytosis and locomotion with-

out marked change in shape.

Infection with poliovirus. The rate of cytopathic change is related to the amount of virus introduced. At first the long processes are withdrawn; the cells become rounded and somewhat granular (Fig. 2). Cinephotomicrographs reveal sudden cytoplasmic vesiculation of some cells which may be due to increased intracellular pressure caused by contraction of the cell membrane and extravasation of cytoplasm through a rupture of the membrane. Later all motion ceases and masses of granular cells with ill-defined boundaries remain (Fig. 3).

Multiplication of poliovirus. Cultivation of poliovirus in CAM cells apparently could be continued for many passages or indefinitely. The titer was lower than that of the original seed virus which had been grown in HeLa cells. This may be due in part to incomplete adaptation of the virus, in spite of 20 passages in the case of strain Y-SK, or to an inherent quality of the CAM cells. Each passage produced a 10-fold dilution of the original inoculum. At the 10th passage the seed virus had been diluted 10⁻¹⁰ and at the 20th passage 10⁻²⁰; yet the titer was only about 2 log units below that of the seed virus, proving that multiplication occurred (Table I). Proliferation of poliovirus was not dependent on the presence of human serum as preliminary studies have shown that the virus

TABLE I. Tissue Culture Titrations of Poliovirus Propagated in Cells Cultured from Chick Embryo Chorioallantoic Membranes.

Test cells	Virus strain	Immuno- logic type	Seed virus	No. of passages in CAM cells				
				5	8	10	18	20
HeLa	Mahoney	1	$10^{-4.5}$	$10^{-3.8}$	$10^{-3.8}$	$10^{-3.2}$		
	Y-SK	2	$10^{-5.2}$		$10^{-3.8}$		$10^{-2.5}$	$10^{-2.8}$
	Saukett	3	$10^{-5.3}$	$10^{-4.9}$	$10^{-3.6}$	$10^{-3.5}$		
CAM	Mahoney	1			$10^{-3.0}$			
	Y-SK	2			$10^{-3.2}$		$10^{-3.0}$	$10^{-3.1}$
	Saukett	3			$10^{-3.5}$			

multiplies also in a strain of CAM cells grown exclusively in mixtures of H, CEE and chicken serum ultrafiltrate. In numerous neutralization tests, type-specific immune serum uniformly gave complete protection and so verified that the cytopathogenic agent in each instance was poliovirus.

Discussion. A fundamental biological difference between certain cells in the intact animal and cell strains derived from them in tissue culture is emphasized by the results of this study. At present no explanation can be offered for the fact that even though poliovirus generally fails to multiply in the unaltered CAM, the strains used infect, multiply in and destroy CAM cell cultures. Reports have appeared on unsuccessful attempts to cultivate poliovirus in chick embryo tissue *in vivo*(4) and *in vitro*(1,5). Roca-Garcia, Moyer and Cox(6) established strain MEF₁ in chick embryos by yolk sac inoculation following passage in suckling hamsters and we (7) obtained multiplication of the Lansing strain for a brief period in cortisone-treated embryos; but these appear to be rare instances of *in vivo* cultivation and in marked contrast to the ready cultivation of poliovirus in CAM cell cultures.

CAM cells may be valuable for cultivation of attenuated strains of poliovirus for human immunization. After establishing a strain of cells apparently free from any other virus, there would be greater security against contaminating virus than when recourse is made to fresh tissue as in the present use of monkey kidney cells. Conversion of the virus to a

strain virulent for humans might be less likely to occur in CAM cells than in human or monkey cells as the CAM cells are derived from a different zoological class.

Summary. Cell cultures were prepared from the chorioallantoic membrane of chick embryos. After 5 transfers these were tested for susceptibility to infection by representative virulent strains of the 3 immunologic types of poliovirus. Multiplication was observed until the series were terminated after 10 or 20 passages. Neutralization tests confirmed the identity of the viruses after passage. The susceptibility of these cells to infection with poliovirus is in marked contrast to that of the tissues from which they were derived.

The authors wish to express their appreciation to Mr. Malcolm Parker for the cinephotomicrographs and Fig. 1 and 2; to Mr. Alex Gravesen for Fig. 3.

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Received April 24, 1957. P.S.E.B.M., 1957, v97.

A Fraction from Liver which Agglutinates Autologous Red Cells.* (23314)

ROBERT R. KOHN AND EDWARD R. ARQUILLA (Introduced by Alan R. Moritz)

Institute of Pathology, Western Reserve University, School of Medicine, Cleveland, Ohio

This investigation was initiated by observation that a homogenate of perfused rat liver would agglutinate red blood cells from the same animal *in vitro*. The substance, or substances, responsible for agglutination was partially purified and its distribution in various species and organs as well as some factors influencing its activity *in vitro* were studied. Evidence is presented suggesting that the agglutinin is a phospholipid.

Methods. Sprague-Dawley rats weighing between 300 and 400 g were used. Rats were anesthetized and bled from the carotid artery into Alsever's solution(3). Erythrocytes were washed several times with isotonic saline solution and diluted to a 2% suspension with isotonic veronal saline buffer (VBS)(3). Organs from the same animal were perfused free of blood with isotonic saline and fractionated as described below. Agglutinating titers of the liver fractions were determined by use of serial dilutions of fractions in VBS containing 0.1% bovine serum albumin. These dilutions were made to final volume of 0.5 ml, to each of which was added 0.05 ml of 2% autologous erythrocyte suspension. The red cells were evenly suspended by shaking and agglutination was determined by observing the pattern of erythrocytes on the bottom of tubes after 3 hours.

Results. After perfusion, rat liver was homogenized in cold acetone and filtered. The insoluble material was dried and extracted with VBS. The extract was dialyzed against distilled water in the cold for 48 hours and the precipitate was separated by centrifugation. Agglutinating activity is precipitated by acetone, extractable by VBS, and precipitates almost quantitatively when dialyzed against water. The titer of the precipitate after dialysis was 1:5120, while that of the supernate was 1:10. On the basis of nitrogen content, dialysis yielded a 500-fold purification.

Effect of heat on activity. Agglutinating activity of the VBS extract of rat liver acetone powder was completely destroyed by heating at 56°C for 30 minutes. However, the precipitate after dialysis did not lose a significant amount of activity after heating at 56°C for 2 hrs. or immersion in boiling water for 5 minutes. Agglutination took place to the same extent when autologous red cells were incubated with liver agglutinin at 6°C, room temperature and 37°C.

Effect of rat serum. High dilutions of both rat plasma and serum completely inhibited agglutination of autologous cells by rat liver fractions. An experiment was carried out in which each tube contained rat liver precipitate after dialysis with N content of 85 μ g (0.2 μ g enough for complete agglutination), plus various dilutions of rat serum. Agglutinations did not occur until serum was diluted 1:200.

Effect of complement. Incubation of the precipitate after dialysis with autologous cells at 37°C in the presence of fresh guinea pig serum resulted in agglutination, but no lysis occurred.

Action on cells of other species. The precipitate after dialysis of rat liver was tested for agglutination of red blood cells of several species. Table I shows that rat liver fraction strongly agglutinates rat, rabbit, and mouse cells, and has a small amount of activity against cells of the dog. Erythrocytes of guinea pig, sheep and human were not agglutinated.

Activity of other organs. Rat kidney, spleen, striated muscle and lung were fractionated as described above for liver and the precipitates after dialysis were tested for agglutinating activity against autologous red cells. Table II shows liver has the most activity, kidney has a slight amount of activity, while spleen, lung and muscle have only traces. Rat spleen could not be perfused free of blood, and it is possible that agglutinating

* Part of this material was presented before the Am. Assn. of Immunologists, April, 1956.

TABLE I. Agglutination of Red Cells of Various Species by Rat Liver Precipitate after Dialysis.

Species of RBC's	Titer of rat liver precipitate
Rat	1/ 5120
Rabbit	>1/20480
Mouse	1/10240
Dog	1/ 160

activity present in spleen was inhibited by serum.

Agglutinating activity of liver from other species. Guinea pig liver was fractionated and the precipitate after dialysis was tested against autologous red cells. Two samples of human liver were obtained at operation and similarly fractionated and tested. Guinea pig liver fraction had a titer of 1:32, while the fractions from human livers had titers of 1:32 and 1:512.

Further identification of the agglutinin. Since various lipid agglutinins have been described, a lipid extraction of rat liver precipitate after dialysis was carried out according to the procedure of Horsfall and Goodner(2). The starting material had a titer of 1:1280, the lipid extract a titer of 1:2560, and the non-lipid residue a titer of 1:10. Thus, the lipid fraction contained essentially all of the agglutinating activity. Four ml of the lipid fraction were evaporated to dryness and analyzed for cholesterol by the method of Kingsley and Schaffert(4). There was no detectable cholesterol in the fraction.

Discussion. Various tissue agglutinins have been described by other workers. Salaman (5) extracted an autohemagglutinin from mouse neoplasms and normal tissues. This agglutinin differed from the one described in

TABLE II. Agglutinating Activity of Various Rat Organs against Autologous Red Cells. Reciprocals of titers were calculated/mg N wet tissue and expressed in arbitrary units.

Organ	Agglutinating activity
Liver	1000
Kidney	6.1
Spleen	3.4×10^{-1}
Lung	8.6×10^{-2}
Skeletal muscle	3.7×10^{-3}

this report by not being found in liver and not being inhibited by serum. Burnet and Stone (1) found various lipid extracts of tissues would agglutinate erythrocytes and that the agglutinating activity was inhibited by serum. However, the extracts would agglutinate only red cells susceptible to agglutination by the vaccinia virus hemagglutinin. Stone(6) in studying agglutinating activity of known lipids found that various lipids agglutinated only red cells susceptible to virus hemagglutination, with the exception of cholesterol ergosterol, and cardiolipin, which also agglutinated non susceptible cells. The agglutinin we described contains no cholesterol, and presumably no ergosterol but it may be related to cardiolipin.

Summary. Homogenates of perfused rat, human and guinea pig livers agglutinate washed autologous red cells. The factor responsible for agglutination was partially purified and it appears to be a heat-stable phospholipid. Agglutination occurred in the cold, at room temperature and at 37°C. Lysis did not occur in the presence of complement. High dilutions of autologous serum completely inhibited hemagglutination. Agglutinating activity was high in liver, present in kidney and practically absent in spleen, lung and skeletal muscle. Rat liver preparations strongly agglutinated red cells of rat, rabbit and mouse, but not cells of dog, sheep, guinea pig or human. Human and guinea pig liver had much less autohemagglutinating activity than rat liver.

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Received April 24, 1957. P.S.E.B.M., 1957, v95.

Absorption and Excretion of Sulfachloropyridazine. (23315)

WILFRED F. JONES, JR., MOHSEN ZIAI, GILBERT R. CHERRICK AND
MAXWELL FINLAND*

*Thorndike Memorial Laboratory, 2nd and 4th (Harvard) Medical Services, Boston City Hospital,
and Department of Medicine, Harvard Medical School, Boston, Mass.*

Interest in the group of sulfapyridazines was stimulated by the finding that one of the derivatives, sulfamethoxypyridazine[†] (SMP) has unusual properties, the most important of which are: good absorption from the gastrointestinal tract, low degree of acetylation in the blood, very slow urinary excretion, good solubility in the urine, low toxicity and antibacterial activity equal to that of the most active of the sulfonamides in popular use(1-7). In this paper it is shown that sulfachloropyridazine[‡] (SCP), which, as seen from Fig. 1, differs only slightly in its structure, is absorbed and excreted quite differently. The methods used were the same as those employed in the study of SMP(1).

Absorption and Excretion of Single Oral Dose of 4 g. Two normal young men were given 4 g of SCP with a glass of water one-half hour before breakfast, after which they were permitted to take food and fluids *ad libitum*. Specimens of oxalated venous blood were taken before and at appropriate intervals after the dose and complete collections of urine were made for 96 hours in one subject and for 24 hours in the other. The concentrations of "free" and "total" SCP in blood, plasma and urines were determined by the method of Bratton and Marshall(8). *Blood levels.* The levels of SCP in whole blood are shown in the upper panels of Fig. 2. The peak concentrations were achieved in both subjects at 3 hours; these values for total SCP were 12.1 mg/100 ml in Subject A and 14.6 mg/100 ml in Subject B, and of these amounts, 10.7 and 11.6 mg respectively, were determined as free drug. Peak concen-

tration in the plasma of Subject A was 21.7 mg/100 ml, of which 2.3 mg was conjugated; the corresponding values in Subject B were 28.5 and 4.9, respectively. The proportion of drug found in the plasma in the conjugated form varied between 15 and 31% (average 23%) in Subject A and between 16 and 33% (average 22%) in Subject B.

Diffusion into blood cells. The concentrations of total SCP in whole blood and plasma were determined in the same specimens and concentrations in the cellular elements were calculated from these values and from the hematocrit. The results indicated that little or none of the drug penetrated the blood cells in Subject A and only a small amount (up to 12% of the plasma level) may have gotten into those of some of the specimens of Subject B when the levels in plasma were high.

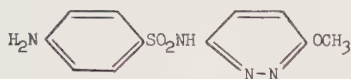
Renal clearances. The rates at which SCP was cleared from the plasma are shown in the lower panels of Fig. 2. The values were estimated without correction for protein binding and during rather long periods and hence are only crude, but they may be useful for comparisons. The conjugated form was cleared on the average about 2½ times more rapidly than the free form, the latter being cleared at rates varying from about 5 to nearly 30 ml of plasma per min.

Urinary excretion. The rapid renal clearance of SCP is reflected in the very high concentrations achieved in the urine during the first few hours after the dose was ingested. As shown in Fig. 3, the urine of Subject A when excretion was at the maximum rate, contained 608 mg per 100 ml, of which 490 mg was free and 118 mg was in the conjugated form. Maximum excretion in Subject B was observed between 3 and 5 hours after the dose and the urine voided during this period contained 667 mg per 100 ml, 502 mg of which was free and 165 mg conjugated. As shown in the upper panels of Fig. 4 maximum

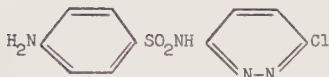
* Aided by a grant from Nat. Inst. of Health. Chemical determinations were carried out by Miss Ellen J. Doyle and Miss Mary I. Kendrick.

† Supplied under the trademark Kynex by Lederle Laboratories Division, Amer. Cyanamid Co.

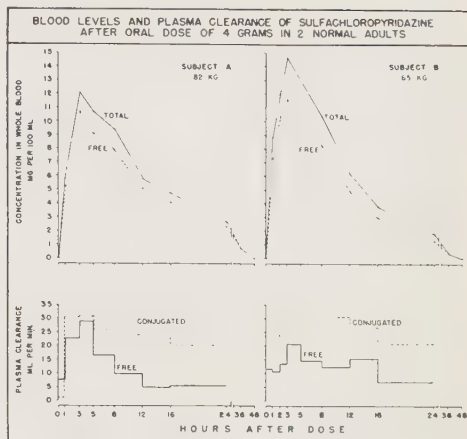
‡ Supplied as BA-10370 by Ciba Pharmaceutical Products.



Sulfamethoxypyridazine (Kynex)

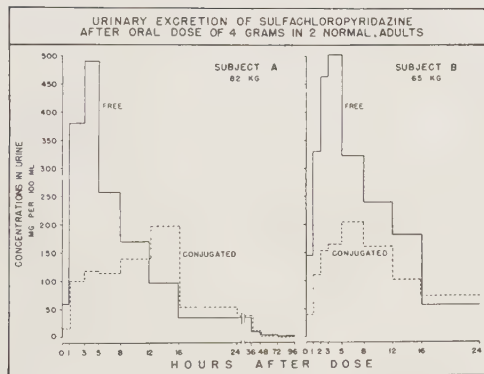


Sulfachloropyridazine (BA-10370)



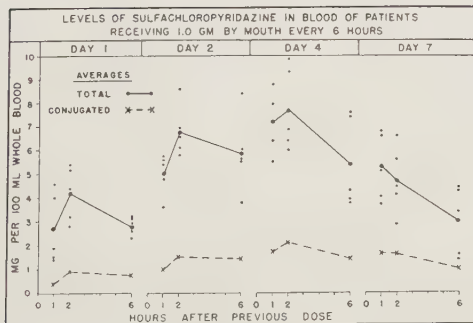
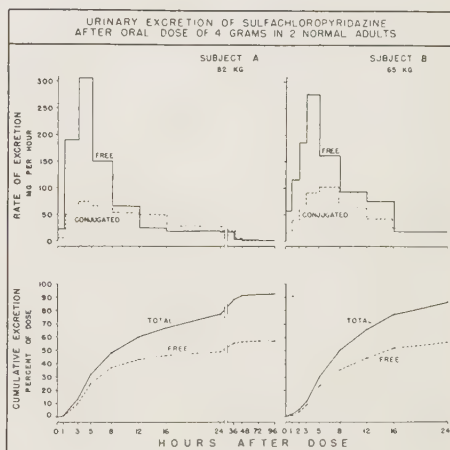
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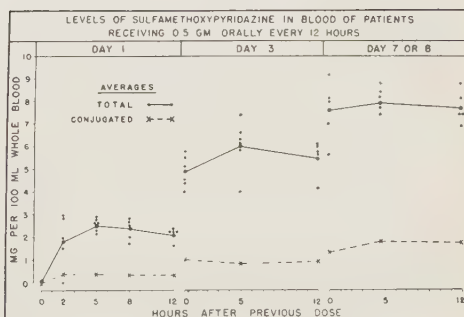


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FIG. 1-6.

TABLE I. Sulfachloropyridazine (SCP) and Sulfamethoxypyridazine (SMP) in Random Specimens of Urine during Continuous Oral Administration.

Drug	Dosage	No. of patients	Day of treatment	Conc. of total sulfonamide in urine, mg %		% of drug conjugated	
				Low	High	Low	High
SCP	1.0 g every 6 hr	5	1	26	248	36	60
		5	2	100	360	43	65
		5	4	106	712	39	61
		5	7	100	738	35	73
SMP	.5 g every 12 hr	5	1	8	79	49	85
		2	2	39	298	51	83
		4	3	19	100	56	74
		3	6	56	363	55	86
		5	8	63	152	54	68

rate of excretion for Subject A was 380 mg per hr (306 free and 74 conjugated) and for Subject B 367 mg per hr (276 mg free and 91 mg conjugated). The cumulative excretions in the 2 subjects, shown in the lowest panels of Fig. 4 indicate that 88% of the administered dose was recovered in the urine of Subject A in 24 hours and only 5% more was recovered during the next 3 days; in Subject B, 86% of the administered dose was accounted for in the urine within 24 hours.

Concentrations of SCP in blood and urine during repeated oral doses. Five patients were given doses of 1 g of SCP orally every 6 hours. They ranged in age from 33 to 57 years and in weight from 64 to 82 (Avg 74) kg. They had no known abnormality of renal function. The findings in the blood are shown in Fig. 5, which depicts the individual levels of total SCP in the whole blood as well as average concentrations of both total and conjugated drug. There were wide variations in blood levels among patients at the different times, but there was a general upward trend in the levels from the first through the fourth day; levels on the seventh day, however, were appreciably lower than on the fourth day. In addition, the highest levels on days 1, 2 and 4 were obtained at 2 hours, whereas on the 7th day they were obtained at 1 hour. The proportion of the drug found in the blood in the conjugated form varied in different specimens from 10 to 50% of the total. There was also a tendency of this proportion to increase as administration of the drug was continued; it averaged about 20% on the first day and 32% on the 7th day. The concen-

trations of drug in random specimens of urine of these patients are listed in the upper part of Table I. Here again there were wide differences in concentration, only part of which were related to the volume of the urine output; the levels of total drug varied from 26 to 738 mg per 100 ml and the proportion of drug found in conjugated form varied from 35 to 73% (Avg 51%).

SMP in blood and urine during continuous oral administration. Six patients were given oral doses of 0.5 g of SMP every 12 hours. These patients ranged in age from 45 to 68 (Avg 52) years and in weight from 60 to 84 (Avg 70) kg; none of them had any recognized abnormality of renal function. Random specimens of urine were also collected from most of these patients. The levels in the whole blood are charted in Fig. 6, which shows the individual levels of the total drug and the average concentrations of both total and conjugated SMP at the various intervals. There was a definite tendency of the drug to cumulate on this dosage during the 7 days of observation, but the levels between doses showed only minor fluctuations. The proportion of drug found in the blood as the conjugated form varied from 12 to 23% of the total and averaged about 17%. The concentration of SMP in random specimens of urine collected from these patients is listed in the lower part of Table I. These levels tended to be considerably lower than those shown for SCP although a concentration as high as 363 mg per 100 ml was noted in one patient. For the most part levels in these urines ranged between 40 and 100 mg per 100

ml for total drug and from 20 to 50 mg per 100 ml of the free form. Conjugation of SMP in the urine occurred to a greater extent than with SCP. The conjugated form accounted for 49 to 86% of the total urinary SMP in different specimens and averaged 62% for all of the specimens.

Discussion. The absorption and excretion of SCP and SMP differ markedly. Both are almost completely absorbed after oral administration of single oral doses, as indicated by the fact that more than 90% of administered doses are accounted for in the urine. The peak concentration of SCP is achieved somewhat sooner (after about 3 hr) than that of SMP (about 5 hr after ingestion of the dose). The half life of a single 4 g oral dose of SCP in the blood, estimated at the time required to achieve one-half the maximum observed concentration in the plasma, is about 11 hours; this is about one-fifth of the half-life of a single 3 or 4 g oral dose of SMP. Whereas one-half of the ingested 4 g dose of SCP can be recovered in the urine within 8 or 9 hours, an average of nearly 60 hours is required to excrete one-half of an oral dose of 3 or 4 g of SMP. These differences are accounted for by the differences in the renal clearance of these drugs, the free SCP being cleared 5 to 20 times faster than free SMP. The conjugated form of SCP is cleared on the average more than twice as fast as its free form, whereas the conjugated SMP is cleared more than 10 times as fast as free SMP. The daily dose required to sustain comparable concentrations of sulfonamide in the blood is about 4 times as great for SCP as for SMP; however, the concentrations of the drugs in the urine from such doses are much greater when

SCP is used. Both on 1 g of SCP every 6 hours and on 0.5 g of SMP every 12 hours there was evidence of cumulation of the drugs in the blood after the first few doses. There was less fluctuation of the levels between these doses of SMP than between the doses of SCP. On doses of 4 g of SCP daily some patients achieve concentrations in the urine which may result in crystalluria and irritation of the urinary tract although none was encountered in the present study. On the average, about $\frac{2}{3}$ of the SMP and $\frac{1}{2}$ of the SCP recovered in the urine after ingestion of these drugs is found in a conjugated form. No toxic effects were observed from either SCP or SMP in any of the patients in this study.

Summary. Sulfachloropyridazine is rapidly absorbed and rapidly excreted in the urine, in contrast to sulfamethoxypyridazine which is also absorbed fairly rapidly but is excreted much more slowly.

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Received April 29, 1957. P.S.E.B.M., 1957, v95.

Host Resistance in Hemorrhagic Shock. IX. Demonstration of Circulating Lethal Toxin in Hemorrhagic Shock.* (23316)

FRITZ B. SCHWEINBURG, PAUL B. SHAPIRO, EDWARD D. FRANK AND JACOB FINE

*Yamins and Kirstein Laboratories, Beth Israel Hospital and Department of Surgery,
Harvard Medical School, Boston, Mass.*

In previous publications of this series evidence was given that hemorrhagic shock inflicts severe damage on the host's antibacterial defense mechanisms(1-6). Impairment of these mechanisms carries a threat to the host if bacteria are already in the tissues, or if they or their toxic products gain access to the circulation during shock. Except for clostridia in the tissues of the dog,[†] most of the tissues and the blood of the dog, rabbit and rat during hemorrhagic shock do not as a rule yield any bacteria by conventional cultural technics. Nevertheless there are data strongly indicating that bacteria or their toxins are operating in hemorrhagic shock, and, if the shock is allowed to persist long enough, that they render the peripheral vessels refractory to transfusion(7-9). In the absence of evidence from cultural data of the presence of bacteria within the blood or tissues, we proceeded to search for a toxin in the circulating blood during shock. A leucotoxin in the plasma of advanced hemorrhagic shock was identified by an *in vitro* comparison of the effect of plasma from normal and shocked animals on phagocytic and bacteriostatic activity of phagocytes(7). To establish the thesis that the refractory state of shock resulting in death is due to bacterial activity, a toxin of bacterial origin, which induces a state of irreversibility to transfusion, must

be identified. The data which follow demonstrate the presence of a lethal toxin in the blood of the dog and rabbit in prolonged hemorrhagic shock, and indicate that intestinal bacteria are the source of the toxin.

Method. Dogs were put into hemorrhagic shock by the elevated reservoir technic(10) and allowed to remain in shock until they became irreversible to transfusion. One or 2 hours after transfusion was shown to be ineffective, *i.e.* when the blood pressure had again fallen below 60 mm of Hg, they were considered preterminal and were exsanguinated. This occurred in nearly every case within 6 hours after inducing shock. The blood so removed, or its plasma, was immediately transfused into a recipient, or stored overnight at 4°C and infused into a recipient the next day. The donor blood or plasma was cultured in every case. With few exceptions which yielded contaminants,[‡] the cultures were found to be sterile. For control data blood or plasma from healthy dogs was treated and used in the same way. The recipients were either healthy dogs, or dogs exposed to severe hemorrhagic shock for 2 hours. They served as test animals for the presence of a toxin in the blood of irreversible shock. Normal dogs can be expected to detoxify a considerable amount of toxin. But dogs in shock for 2 hours, which recover when transfused with normal blood or with their own shed blood at 2 hours, might not recover if transfused at

* Aided by a grant from Natl. Heart Inst., N.I.H., Bethesda, Md., and Contract with Office of Surgeon General, through Subcommittee on Shock of Natl. Research Council.

† Clostridia in the dog's tissues are considered not to be of primary importance because (1), polyvalent clostridial antitoxin in large doses is of no prophylactic or therapeutic value; (2), neomycin was among the most effective antibiotics which prevented development of irreversibility to transfusion(8), and (3), as will appear below, non-absorbable antibiotics given orally in advance of producing shock prevent appearance of a lethal toxin in the blood of dogs in advanced shock.

‡ Three of 12 donor bloods yielded a coagulase negative, non-hemolytic *Staphylococcus albus* in 2 *Pseudomonas fluorescens* n. 1. in one, and *B. subtilis* in one. The same bacteria were found in the skin surrounding the groin incision made for cannulating the femoral artery. Toxicity of these bacteria was tested by intravenous injection of 100 million bacteria into dogs during shock. These dogs were transfused after 2 hours in shock. Their blood cultures were sterile the next day and they recovered with no apparent injury.

this time with donor blood or plasma containing a toxin, because their defenses are down. In some cases the entire volume of donor blood obtained from a single dog was given to a single recipient. When the recipient was a normal dog, a volume of blood equal to the volume of blood or plasma to be transfused was removed just prior to the transfusion. When the recipient was a 2 hour shock dog, the volume of donor blood given never exceeded the blood volume deficiency of the recipient. When the volume of donor blood given was below the blood volume deficiency of the recipient, the latter received enough of his own shed blood to restore the normal blood volume. In some experiments 3 or 4 two-hour shock recipients were tested simultaneously with different fractional volumes of the blood from one donor, in order to determine its lethal potency. In these, as in the other experiments, the recipient received the total volume of blood in the reservoir, *i.e.* his own shed blood, minus the volume of donor blood administered at the same time. When plasma instead of whole blood was infused, the recipient received his own shed red cells instead of his own shed whole blood as a supplement. In some of these instances, therefore, the normal blood volume was not fully restored, but the deficiency was not sufficient to prevent full recovery. The foregoing experiments were also performed on rabbits, except that only whole blood was used as the test substance, and, in the case of the shock recipient, the donor blood was given after 1½ instead of 2 hours of shock. Additional experiments were performed with donor blood from dogs which received non-absorbable antibiotics orally and by rectum prior to inducing shock. The antibiotic therapy was as follows: bacitracin (200,000 U) and polymyxin B (200 mg) were given daily for 3 days. They were buried in food, with care to see that the food was totally consumed. On the next (fourth) day, 3 hours before inducing shock, 200,000 U of bacitracin and 600 mg of polymyxin B in 200 ml of saline were given by gavage, and 200,000 U of bacitracin and 100 mg of polymyxin B were given in normal saline by proctoclysis. We have elsewhere (8) noted that dogs receiving prophylactic

antibiotic therapy often display greater than normal resistance to prolonged severe hypotension. In the present series of experiments many of the dogs pretreated with antibiotics were still compensating, *i.e.* they were not taking blood back from the reservoir even after 6 hours in shock. All these dogs were transfused at this time. Their response to transfusion was in most cases good, and they were exsanguinated 15 to 20 minutes after transfusion. The blood from these dogs was tested for toxicity by infusion into 2 hour shock recipients.

The above experiment was repeated in 3 groups of 5 rabbits each, except that only one antibiotic was administered to each animal and none was given by rectum. All 15 rabbits receiving bacitracin (10,000 U daily for 4 days), polymyxin B (100 mg for 3 days and 200 mg on fourth day) or neomycin (100 mg for 3 days and 200 mg on fourth day) were kept in shock for 6 hours. In our experience rabbits not pretreated with antibiotics almost never tolerate hemorrhagic shock for this length of time. The pretreated rabbits remained fully compensated throughout this period, *i.e.* none took back more than a few ml of blood from the reservoir. Transfusion was then performed, and after some thirty minutes they were exsanguinated. Rabbits in shock for two hours were transfused with these bloods instead of their own shed bloods.

Results. (Table I). I. Five normal dogs received whole blood from irreversibly shocked dogs immediately after removal of an equal volume of their own blood (500 to 1000 ml). They survived without noticeable harm.

II. Fifty dogs were transfused with their own shed blood after 2 hours in severe hemorrhagic shock. These dogs were able to stand and take fluids within an hour or 2 after the transfusion. Two died and 48 (96%) recovered rapidly and uneventfully.

III. Twenty-six dogs were transfused after 2 hours in severe hemorrhagic shock with blood from dogs dying of irreversible hemorrhagic shock. The volume of donor blood varied from 50 to 1078 ml. Eight (31%) survived. Of the 8 survivors 6 were very sick, exhausted, took little food or fluid, and

TABLE I. Effect of Whole Blood or Plasma from Animals in Advanced Shock on Survival Rate of Animals in Reversible Shock.

		Survival rate (%)	
Amt and type of test substance	No. exp.	Normal recip- ients	2 hr shock re- cipients
Dogs			
Whole normal blood	20		90
Whole blood (R)*	50		96
" " (I.S.)†	26		31
" " (I.S.)	5	100	
Plasma (I.S.)	10		20
Rabbits			
Whole blood (R)	50		90
" " (I.S.)	12		8
" " (I.S.)	4	100	
Pretreatment of donors with non-absorbable antibiotics			
Whole blood (P.S.)‡	16 (dogs)		63
" " (P.S.)	15 (rabbits)		93

* R = Recipients' own shed blood standing in reservoir for 2 hr.

† I.S. = Irreversible shock. Donor blood obtained by exsanguination following ineffective transfusion for shock of 4 or 5 hr duration.

‡ P.S. = Prolonged shock. Donor blood obtained by exsanguination following transfusion for shock of 6 hr duration.

displayed a bloody diarrhea for several days. All those which died were very sick, prostrated, took no food or fluid and showed bloody diarrhea from the time of transfusion until death. Of the 26 recipients in this series, 19 dogs were divided into 5 groups, with 3 or 4 dogs per group. Each group was tested for toxicity of the whole blood from one irreversible shock donor (Table II). The volume infused into a single recipient varied from 50 to 600 ml. Of the 19 recipients only 5 survived. Three of them were in one group of 4 which had received 50, 100, 200 and 400 ml respectively of the same donor blood. Only the recipient of the 400 ml fraction died. It was the longest survivor among the 14 which died. Most of the recipients died within 24 hours.

IV. Ten dogs were transfused after 2 hours in severe hemorrhagic shock with their own shed red cells together with plasma from dogs dying of irreversible hemorrhagic shock. Two (20%) survived. Of the 10 plasmas given, 5 had been Seitz-filtered. Of these 4 were lethal in volumes varying from 150 to 200 ml. The survivor of the fifth plasma was

very sick with bloody diarrhea for 3 days. Of the 5 which were not Seitz-filtered, 4 were lethal in volumes varying from 200 to 500 ml. The survivor of the fifth showed no signs of injury. The 8 recipients of plasma which died manifested the same disorder after transfusion as those which died after receiving whole blood from shocked donors.

V. Sixteen dogs were transfused after 2 hours in severe hemorrhagic shock with blood (350-800 ml) from dogs in prolonged hemorrhagic shock after pretreatment with non-adsorbable antibiotics. Ten (62.5%) survived. It is noteworthy that 8 of the survivors were free of any overt signs of illness, and recovered with the same rapidity as 2 hour recipients receiving their own shed blood. The other 2 survivors were slightly ill for 24 hours without diarrhea.

VI. Four normal rabbits received whole blood (33-54 ml) from rabbits dying of irreversible shock after removal of an equal volume of their own blood. All survived without noticeable harm.

VII. Twelve rabbits were transfused after 1½ hours in severe hemorrhagic shock with whole blood (32-60 ml) from rabbits dying of irreversible shock. One (8.6%) survived. The remainder were prostrate and showed

TABLE II. Titration of Lethal Potency of Blood from Irreversibly Shocked Dogs in 2 Hour Shock Recipients.

Exp. No.	Donor blood (ml)	Own blood (ml)	Result*
I	100	900	D 24 hr
	200	1000	D "
	600	500	D "
II	50	1090	S
	100	1280	S
	200	1000	S
	400	350	D 72 hr
III	75	1175	D 24 hr
	200	650	D "
	300	930	D "
	500	210	D "
IV	75	1025	S
	200	650	D 18 hr
	300	650	D "
	500	450	D 24 hr
V	50	1120	D 24 hr
	100	800	D "
	300	920	S
	450	550	D "

* D = Death; S = Survivor.

bloody diarrhea until they died. Death occurred within 24 hours.

VIII. Of fifteen rabbits transfused after $1\frac{1}{2}$ hours in severe hemorrhagic shock with whole blood (40 ± 12 ml) from fifteen rabbits in shock for six hours, but pretreated with bacitracin and polymyxin B or neomycin, only one died. Fourteen responded promptly and recovered without any symptoms.

Comment. These results demonstrate the presence of a lethal substance in the blood of dogs and rabbits in advanced hemorrhagic shock. Since the infused blood or plasma was sterile on culture, and since Seitz-filtered plasma was as lethal as unfiltered plasma, this substance is not bacteria, but a toxin which the animal in shock cannot detoxify. Current work, to be reported later, indicates that this toxin is an endotoxin.

In a previous publication(5) a lethal substance was shown to be present in the liver of shocked dogs. Because penicillin given prophylactically prevented death, we concluded that the lethal substance was bacteria. This conclusion became dubious when subsequent data(6) showed that an antibiotic given prophylactically reduces the lethality of endotoxin. The explanation given for this was that endotoxin injures the defenses against bacteria, so that if a dose of endotoxin is itself sublethal, the unopposed action of bacteria will then produce endotoxin, which, added to the amount administered, may constitute a lethal dose.

That the toxin is present not only in blood and liver, but in other tissues and fluids of shocked animals, is evident from experiments now in progress showing that the lymph, lung and spleen also contain a toxin with properties like those of the toxin in the blood. Since the toxin is widely distributed throughout the body, it is understandable that an animal in shock cannot be detoxified by exsanguination transfusions. On the other hand, cross-circulation of the shocked animal with a healthy donor, performed so as to protect a tissue that neutralizes toxins, *e.g.* liver(11), prevents irreversibility to transfusion.

For reasons given elsewhere(13,14), we believe that endogenous toxins that might be generated in consequence of the shock

process *per se* do not account for irreversibility and death. This view is confirmed by the experiments with non-absorbable antibiotics. Since these antibiotics could not have prevented development of such endogenous toxins,[§] but reduced mortality rate of vulnerable recipients (dogs and rabbits) from 81% to 24% (Table I), it follows that the toxin is derived from the activity of intestinal bacteria and is, therefore, exogenous. This conclusion is consistent with the observation that irreversibility to transfusion and death can be prevented by cross-circulation of the shocked dog with a normal donor dog *via* the superior mesenteric artery(12).

The hemorrhagic lesion in the gut of the shocked animal is due to the toxin, and not to the shock itself. This is manifest from the response of 2 hour shock recipients of blood from other animals in advanced shock. Thus, animals in shock for 2 hours recover without intestinal damage when transfused with normal blood, with their own shed blood, or with blood from donors pretreated with antibiotics. But nearly all 2 hour shock recipients of blood or plasma from untreated donors in advanced shock, whether they survive or die, develop a bloody diarrhea soon after the transfusion, and show at post-mortem a swollen hemorrhagic mucosa as well as intramural hemorrhage.

Since as much as a liter of blood from an animal in advanced shock does not kill a normal animal, whereas as little as 50 ml may kill an animal in shock of 2 hours duration, it follows that the major difficulty in the latter is the collapse of the detoxifying mechanisms. That this collapse occurs early in shock can be seen from the following experiment: Ten dogs were transfused after 2 hours in shock and exsanguinated after the maximum pressor response. Ten other dogs were transfused with these bloods instead of with their own shed blood after shock of 2 hours duration. Three of the latter were noticeably weak for the next 18 hours, and then rapidly

[§] The same applies to exotoxins from bacteria in the tissues. Since the donor blood was in all instances free of hemolysis, clostridial exotoxins in particular, whether formed in the gut or tissues, would appear not to be involved.

recovered. The remaining seven were very sick immediately after the transfusion and displayed bloody diarrhea. Of the 7, two began to recover after 36 hours; the other 5 died during the next two days. Since their own shed blood would not have caused a hemorrhagic lesion in the gut or death, it is clear that the donor blood was toxic, whereas their own shed blood was not. (Shed blood is not toxic because it is normal blood released into a bottle in order to produce shock, and has remained out of contact with the circulation until reinfused.) This experiment identifies a toxin in the blood of animals during the first two hours of shock. Although the shock is still reversible, the detoxifying mechanisms are already failing.

Work in progress demonstrates that a toxin with the same properties as the toxin present in the blood of hemorrhagic shock is also present in the blood of animals in other types of shock.

Summary and conclusions. I. Normal dogs tolerate an infusion of blood from dogs dying of advanced hemorrhagic shock without noticeable harm. Dogs in hemorrhagic shock recover rapidly and without signs of illness if transfused after 2 hours with normal donor blood, or with their own shed blood. But about 70% of such dogs die if the transfused blood is from dogs dying of prolonged hemorrhagic shock. Most of the recipients that survive, as well as all those that die, are prostrated, unable to stand, drink or take fluid after the transfusion, and display a bloody diarrhea until recovery or death. Only 37% of the 2 hour shock recipients of blood from dogs in prolonged shock die if the latter have been vigorously pretreated with non-absorbable antibiotics given orally and by rectum. The survivors, moreover, recover rapidly and disclose no signs of illness. Hence the blood of dogs in prolonged hemorrhagic shock contains a lethal toxin, which, for the reasons given, is considered to be an exogenous toxin derived from the intestinal flora. By the same token en-

dogenous toxins, whether present or not, are not responsible for irreversibility to transfusion or death. II. The same findings are also true for the rabbit: The mortality rate of recipients of blood from untreated donors was 91%, while the mortality rate of recipients of blood from pretreated donors was only 7%. The greater tolerance to prolonged shock of animals pretreated with non-absorbable antibiotics appears to be due to the elimination of bacterial endotoxins by the antibiotics. III. The hemorrhagic lesion in the gut of shocked animals is produced by the toxin and not by the shock itself.

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Received April 29, 1957. P.S.E.B.M., 1957, v95.

Failure to Transmit Common Cold to Suckling Hamsters.* (23317)

WILLIAM S. JORDAN, JR.[†] AND FLOYD W. DENNY, JR.

*Departments of Preventive Medicine, Medicine, and Pediatrics, School of Medicine,
Western Reserve University, and University Hospitals, Cleveland, O.*

Reagan *et al.*(1,2) have reported the successful transmission of the common cold to 6-day-old suckling hamsters by the intranasal instillation of filtered saline nasal and throat washings from individuals with acute coryzal illnesses. Such material from each of 4 donors induced "cold virus signs, such as running nose and wheezing, with the nostrils swollen, boggy and inflamed"(1). Attack rates ranged from 50 to 100%; incubation periods varied from 3 to 7 days(2). The investigators reported that instillation of material from the fourth hamster passage, while inducing typical colds in nonimmune hamsters, failed to do so in animals having recovered from previously induced infections. Instillation of filtered washings from a normal person did not induce signs in control litters. Interest in these results was heightened by information regarding ailments of hamsters provided in several hamster manuals. One devoted a section to "colds in the hamstery," and stated that the common cold "can travel through a hamstery like distemper through a fox or mink yard . . . A first precaution is to abstain from handling hamsters while you yourself have a cold."(3). The manifestations of the infection were described as inactivity, swollen nose, fur ruffled from constant wiping of nasal discharge, sniffing and sneezing.

The present report summarizes experiments undertaken to confirm the above observations, and records the failure of inocula infectious for man to induce disease in suckling hamsters.

Materials and methods. Inocula. The

* This investigation was conducted under the sponsorship of Commission on Acute Respiratory Diseases, Armed Forces Epidemiological Board, and supported in part by Office of The Surgeon General, Department of the Army, and by grants from Brush Fil., Robert Hamilton Bishop, Jr. Endowment Fund, and Republic Steel Corp.

[†] Markle Scholar in Medical Science.

nasal secretions used in Exp. 1 were blown into Petri dishes by 2 donors with acute coryzal illnesses. One secretion (JMR) was diluted 1:5 in maintenance mixture (7.5% chicken serum, 25% tryptose phosphate broth, 67.5% synthetic maintenance fluid)(4) and stored at -70°C for 3 months; the other (WSJ) was diluted 1:2 in tryptose phosphate broth and stored at -70°C for about one year. Prior to use, phenol red was added to the second specimen and the pH adjusted so that it could not be distinguished from maintenance fluid. The inocula used in Exp. 2 were all provided under code by Dr. George G. Jackson. Except for C, Hanks' salt solution, they all were derived from N.S. #142. This secretion was collected by Jackson from a young adult with a coryzal illness on March 6 and 7, 1956, and stored at -70°C until Apr. 20, 1956. On this date it was thawed, diluted 1:10 with Hanks' balanced salt solution containing 5% yeast extract, and passed through a Swinney filter. The filtrate was dispensed into screw cap vials, and stored at -70°C . In 7 experiments between Apr. 1956 and Feb. 1957, Jackson(5) induced colds in 62 of 122 volunteers by intranasal instillation of aliquots of this secretion. On Nov. 30, 1956, 8 days before hamster experiment 2, an aliquot diluted 1:100 produced colds in 4 of 12 volunteers. On Feb. 8, 1957, 2 months after that experiment, another aliquot induced colds in 5 of 15 volunteers. The material used on November 30 was twice filtered, and the unused portion was refrozen and coded as inocula A and B. Inocula coded X and Y were aliquots of the original 1:10 dilution. Inoculum Y was further diluted (final 1:20) in this laboratory with an equal volume of maintenance fluid. Inoculum Z represented material that had been refrozen after use in an experiment in which 50% of the volunteers developed colds. In Exp. 3, a secretion (JHD) was used that had been stored at -70°C for 6 years. On 2 separate occasions in 1950, this

TABLE I. Attempts to Transmit Common Cold to 6-Day-Old Suckling Hamsters.

Exp. No.	Inoculum	Litter No.	No. in litter	Deaths†	
				No.	Day post inoculum
1 11/27/56	Nasal secretion JMR, 9/5/56; 1:5 in maintenance mixture	1	5	0	
		2	5	2	3, 4
		3	7	3	1, 6, 6
	None	4	10	2	5, 8
	Maintenance mixture	5	13	3	All on 8
		6	8	2	8, 9
		7	7	0	
	None	8	6	0	
	Nasal secretion WSJ, 1/4/56; 1:2 in tryptose phosphate broth	9	10	1	10
		10	10	10*	All on 9 and 10
		11	9	9	4 on 7; 5 on 10
	None	12	5	0	
2 12/ 8/56	N.S. #142; 1:100 in Hanks' BSS c 5% yeast extr.; twice filtered; 2 samples	A 13	7	0	
		14	7	0	
		B 15	9	0	
		16	11	0	
	Hanks' BSS	C 17	6	2	1, 1
		18	8	0	
	N.S. #142; 1:20 in Hanks' BSS c 5% yeast extr.	Y 19	7	0	
		20	11	2	12, 12
	Same as "Y" above—heat inactivated	X 21	8	1	1
		22	7	0	
	N.S. #142; 1:100 in Hanks' BSS c 5% yeast extr.	Z 23	6	0	
		24	10	0	
3 1/26/57	Nasal secretion JHD, 2/3/50; 1:10 in milk	25	6	0	
		26	4	0	
	Sterile skim milk	27	7	0	
		28	3	0	
	Maintenance mixture	29	4	2	1, 4
		30	7	0	
	Nasal secretion CF, 12/7/56; in maintenance mixture	31	8	0	
		32	6	0	

* Mother tunneled under barrier and deserted litter on day 9.

† None of these hamsters had nasal signs.

secretion, diluted 1:10 in skim milk, had induced colds in 50% of volunteers. The other secretion (CF), from an individual with a moderately severe cold, was collected on nasal swabs, placed in 2 ml of maintenance fluid and stored at -70°C for 7 weeks prior to use. *Hamsters.* The litters were shipped by dealer 2 days after delivery, and received in the laboratory on the fourth day. The mothers and sucklings were left in the same $9\frac{1}{2} \times 12\frac{3}{4}$ in. fruit can in which they had been shipped. In Exp. 1, there were 2 litters in each can separated by a solid partition. In Exp. 2 and 3, there was 1 litter per can. After coding, the inocula were dropped on the

noses of 6-day-old unanesthetized sucklings from a No. 26 or 27 gauge needle, approximately 0.03 ml/suckling. In most instances, the 3-4 small drops were inhaled by the hamster. The animals were then observed daily for development of nasal signs.

Results. Table I shows the details of the 3 experiments. In all, 138 suckling hamsters in 18 litters were given potentially infectious materials. Five (A, B, Y, Z, JHD) of the 8 inocula had induced colds in human volunteers. Control inocula were given to 78 hamsters in 11 litters. Twenty-one sucklings in 3 litters were not inoculated. None of the hamsters developed nasal signs or obvious respira-

tory symptoms during 10 to 12 days of observation. Deaths occurred in both the infected and control litters. The majority of deaths occurred during the first experiment when the animals were housed in crowded quarters and the bedding was allowed to become wet. In one instance (litter No. 10) the mother deserted the sucklings. Avoidance of crowding in Exp. 2 and 3 limited non-specific deaths to the runts of the litters. None of the animals exhibited swollen nostrils, coryza, wheezing, or ruffled fur.

Discussion. The inocula used in these experiments had been stored at -70°C for varying periods of time, introducing a consideration of the stability of infectivity of the common cold virus at this temperature. Andrewes(6) found that filtrates of nasal secretions suffered no loss of infectivity when so stored for 2 years. Feller *et al.*(7) induced colds in volunteers with material that had been stored for 16 months. With 5 different secretions, Jackson(5) noted no loss of infectivity during periods of storage of from 40 to 49 weeks. While parallel transmission experiments were not done simultaneously in

both humans and hamsters, these considerations plus the evidence regarding the infectiousness for humans of N.S. #142 both before and after experiment 2, justify the conclusion that the negative results reported here are valid.

Summary. Nasal secretions from 5 different individuals with coryzal illnesses were inoculated intranasally in 138 suckling hamsters. No evidence was obtained to indicate that human colds can be transmitted to suckling hamsters.

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Received April 29, 1957. P.S.E.B.M., 1957, v95.

Blood Levels of 17-Hydroxycorticosteroids (17-OHCS) During Labor of Human Pregnancy.* (23318)

ERIC MCKAY,[†] N. S. ASSALI AND MARGARET HENLEY

Department of Obstetrics and Gynecology, University of California at Los Angeles and the Los Angeles Harbor General Hospital

It is now generally accepted that the activity of the adrenal cortex is increased in pregnancy(1-8). Blood levels of 17-OHCS have been found to increase progressively throughout the course of gestation reaching its maximum at the end of gestation and returning to low levels one week after delivery (2,3). These levels were higher in the primipara than in the multipara and equally higher in patients having vaginal deliveries than in

patients undergoing Cesarean section(3,9,10). These differences were attributed to the stress of labor which is more severe in primipara and during vaginal deliveries. This assumption received support from observations of high plasma levels of these hormones at the time of umbilical cord ligation(11).

The present study was aimed at assessing the effects of the various stages of labor and delivery on blood levels of 17-OHCS. This was done by following the same group of patients throughout labor until approximately one week after delivery.

Material and methods. Thirty-four pregnant subjects whose antenatal courses had

* This study was supported by grants from the Nat. Heart Inst. and the Los Angeles Harbor General Hospital Research Fund.

[†] Present address: Royal Women's Hospital, University of Melbourne, Australia

been uneventful were selected at random. Ten patients were in the last trimester but not in labor and served as controls. Twenty-four patients were studied throughout their labors, deliveries and postpartum periods. Three periods during parturition were chosen for study: the first period encompassed the first stage of labor, the second period referred to the second stage of labor, and the third period represented the actual time of delivery. In addition, blood samples were taken at 30 minutes following delivery and on the 4th or 7th day postpartum. Although nearly all patients had samples taken when admitted early in labor, no more than 2 to 4 further samples were taken from any one patient. The methods for collecting blood samples and determining the concentration of 17-OHCS in the plasma have been described previously (8). Most patients were given 1 to 3 injections of intramuscular Demerol (100 mg) as required for analgesia, while anesthesia for delivery was usually local or pudendal block, with nitrous oxide and oxygen inhalation. Spinal anesthesia was given in a few cases. Two patients, Nos. 16 and 19 were delivered by Cesarean section.

TABLE I. Plasma Levels 17-Hydrocorticosteroid in Various Stages of Labor and Postpartum in 23 Patients.

1st stage	2nd stage	Delivery	½ hr post-partum	4th day post-partum
30.0	65.6	80.2	62.8	45.3
88.3		173.3	69.4	
	36.8			37.7
31.0		55.6		
67.5	115.0			64.2
67.0		83.4		25.8
72.6	75.9			61.1
29.5	58.7		46.6	37.9
33.1				
30.5	95.6			37.2
71.2	58.9			
33.9		82.9	43.5	22.0
79.8		77.2		23.7
59.6	111.4			22.0
19.2	78.6			
*		34.7		
78.6	98.3		105.0	
43.3		47.6		27.6
* 44.8				
84.4		127.1		40.6
65.8	79.0			
	70.4			
53.9		69.3		19.2

* Cesarean section.

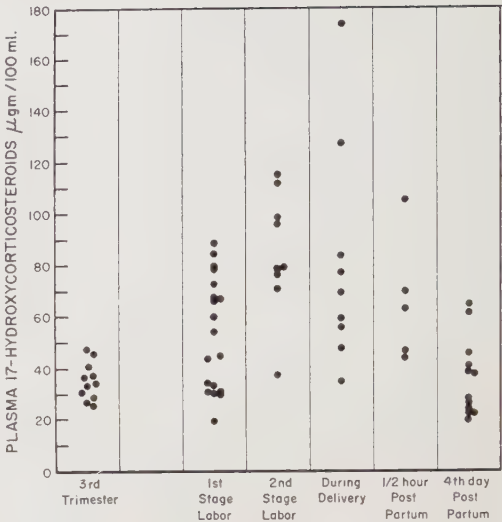


FIG. 1. Distribution of plasma volumes for 17-OHCS during last trimester of pregnancy (not in labor) and in various stages of labor and after delivery.

Results. Detailed data on blood levels of 17-OHCS during various stages of labor and after delivery are listed in Table I. Fig. 1 compares these values to those of a control group in the last trimester but not in labor. The values for plasma 17-OHCS in the control group not in labor ranged from 25.6-47.5 µg% with a mean value of 34.6 µg%. These levels compare well with previous studies(2, 3,5-8) and are well above nonpregnant values of 7 to 15 µg%.

In the first stage of labor, plasma levels ranged from 19.2 to 88.3 µg% with a mean of 50.6. In the 2nd stage of labor the values ranged from 36.8 to 115 with a mean of 80.3 µg%. The wide variation observed was probably due to differences in the reaction of patients to labor, duration of labor and some other subjective variables which could not be easily controlled. At the time of delivery, the values ranged from 34.7 to 173 µg%. The highest value was observed in a patient with possible liver disease. Immediately after delivery the plasma levels of 17-OHCS were still high, ranging from 43 to 105 µg%. Four days postpartum the levels had fallen somewhat but were still higher than those of nonpregnant patients (7-15 µg%).

Statistical analyses of the data showed that the difference between the values obtained

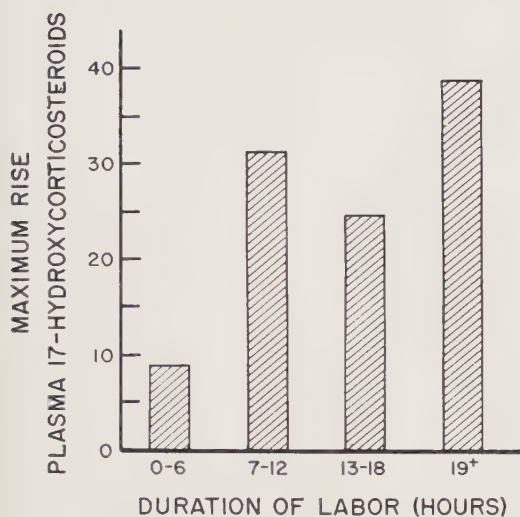


FIG. 2. Plasma levels of 17-OHCS in relation to length of labor. Labors lasting less than 6 hr had significantly lower values than labors lasting longer.

during labor and those obtained from the control series not in labor was significant ($P < .01$); the difference between first and second stage of labor was also significant ($P < .05$); and so was the difference between the values obtained during labor and those of the postpartum period ($P < .01$). Plasma levels of 17-OHCS were lower in patients whose labor lasted less than 6 hours (Fig. 2). Beyond 12 hours of labor there was little further increase in values.

Discussion. The data indicate a variable but consistent rise in plasma levels of 17-OHCS during parturition. Maximum elevation occurred at the stage of expulsion of the fetus.

Although mean values at the fourth or seventh postpartum day were below the initial values for patients in labor, they were in most cases well above normal. This could be caused by either a faulty metabolism of these hormones during the postpartum period or by the fact that the stimulus responsible for elevation of plasma levels of 17-OHCS during parturition had not subsided by the end of the first week after delivery.

The source of the excess of 17-OHCS observed during labor is difficult to determine. It has been shown previously(12) that the elevated levels of pregnancy were due to ex-

cessive production rather than to faulty metabolism of these hormones. It was further shown that despite the already elevated levels in normal pregnancy, the adrenal cortex was still capable of responding to additional stimulation with exogenous ACTH.

In view of the rise in 17-OHCS observed in the last stage of labor approximating those found after maximum ACTH stimulation in the last trimester of pregnancy(12) and in patients with Cushing's disease under similar stimulation(13), it is possible that labor imposes a maximum stimulus upon the adrenal cortex of the mother. This, however, does not rule out the possibility that the placenta might contribute in some way or another to these high levels.

The mechanisms by which labor could stimulate the adrenal cortex are obscure. Assuming that the maternal pituitary gland is the major site of adrenocorticotrophic hormones in pregnancy, it is probable that labor stimulates this gland to increase its release of ACTH. Several workers(14,15,16) have observed that certain posterior pituitary hormones may stimulate ACTH secretion. Martine *et al.*(17) have claimed that both vasopressin and oxytocin may act in this way, while others(18) have failed to confirm any significant action of either pitocin or syntocin in animals. If oxytocin stimulates ACTH release in human subjects, then it is reasonable to accept the idea that labor stimulates the pituitary gland of the mother through the action of oxytocin. This does not rule out the contribution of the stimulus provoked by the pain itself and by the muscular work involved in labor since these factors *per se* have been shown to lead to increased plasma levels of 17-OHCS(19,20). The contribution of each of these variables is now being studied.

Summary. 1. Blood levels of 17-OHCS were studied at intervals during parturition in normal pregnancy. 2. Early in labor, a rise in levels above those found in late pregnancy was noted, with further rise between first and second stages. These high levels were maintained until after the end of 3rd stage. In most cases studied, levels were still well above normal on 4th or 6th postpartum day. 3. There seems to be a relation between

length of labor and rise in plasma 17-OHCS. Labors of longer than 6 hours deviation were accompanied by high levels of these hormones. 4. Maximum values observed in labor approached those noted after exogenous ACTH stimulation during late pregnancy, indicating nearly maximal output of these hormones by the adrenal cortex. 5. The mechanisms by which labor stimulated the maternal pituitary gland have been discussed.

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Received April 30, 1957. P.S.E.B.M., 1957, v95.

Beneficial Effects of Alfalfa and Other Succulent Plants on Glucoascorbic Acid Toxicity in the Rat.* (23319)

BENJAMIN H. ERSHOFF

Department of Biochemistry and Nutrition, University of S. California, Los Angeles

Woolley and Krampitz(1) have observed that immature mice fed a purified ration containing 5 to 10% glucoascorbic acid developed a severe condition characterized by failure of growth, diarrhea, subcutaneous hemorrhages, irritation and swelling of the anal region, alopecia and death. These effects were completely counteracted by concurrent feeding of dried grass(1) or alfalfa meal(2). Supplements of the known nutrients, in contrast to results obtained above, were without significant effect(2). In the present communication data are presented on comparative

effects of alfalfa and other materials of plant and animal origin on glucoascorbic acid toxicity in the rat.

Procedure. The basal ration consisted of sucrose, 62%; casein,[†] 24%; salt mixture,[‡] 5%; corn oil, 5%; and glucoascorbic acid,[§]

[†] Vitamin-free Test Casein, General Biochemicals, Chagrin Falls, O.

[‡] Hubbel, Mendel and Wakeman Salt Mixture, General Biochemicals, Chagrin Falls, O.

[§] The glucoascorbic acid was prepared by procedure described in U. S. Patent No. 2,206,374. The author is indebted to Dr. Robert Irwin for synthesizing this material and to Mr. Philip P. Gray of Wallerstein Laboratories, N. Y. City, for permission to employ patent procedure and his helpful suggestions in preparation of this product.

* Communication No. 434 from Dept. of Biochemistry and Nutrition, Univ. of S. Calif. This investigation was supported in part by grant-in-aid from Nutrilite Products, Inc., Buena Park, Calif.

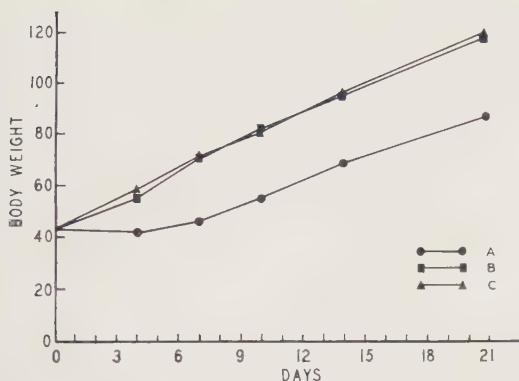


FIG. 1. Weight increment of rats fed glucoascorbic acid with and without alfalfa meal. Diet A, basal ration with 4% glucoascorbic acid; diet B, basal ration with 4% glucoascorbic acid plus 20% alfalfa meal; diet C, basal ration with glucoascorbic acid omitted. Each group consisted of 10 rats. Three rats fed diet A died during first week of feeding; all remaining animals survived experimental period.

4%. To each kg of above diet were added the following vitamins: thiamine hydrochloride, 10 mg; riboflavin, 10 mg; pyridoxine hydrochloride, 10 mg; calcium pantothenate, 60 mg; nicotinic acid, 100 mg; ascorbic acid, 200 mg; biotin, 4 mg; folic acid, 10 mg; para-aminobenzoic acid, 400 mg; inositol, 800 mg; vit. B₁₂, 150 μ g; 2-methyl-naphthoquinone, 5 mg; choline chloride, 2 g; vit. A, 5000 U.S.P. units; vit. D₂, 500 U.S.P. units; and alpha-tocopherol acetate, 100 mg. The vitamins were added in place of an equal amount of sucrose. Thirty female rats of the Univ. of S. Calif. strain were selected at 22 to 25 days of age and average body weight of 43.3 g (range 40 to 46 g). Animals were divided into 3 comparable groups of 10 rats each and were fed the following diets: (a) basal ration, (b) basal ration plus 20% alfalfa meal and (c) basal ration with glucoascorbic acid omitted and replaced by equal amount of sucrose. Animals were placed in metal cages with raised screen bottoms and were fed above diets *ad lib*. Animals were fed daily and all food not consumed 24 hours after feeding was discarded. All diets were stored in refrigerator to minimize oxidative changes in the diet. Feeding was continued for 21 days or until death whichever occurred sooner. Weight increment of rats on the various diets is indicated in Fig. 1. For the first

7 days of feeding, animals fed the basal ration exhibited a significant retardation in growth. They developed extensive diarrhea, varying degrees of alopecia and unthrifty appearance, with death occurring in 30% of the animals. Thereafter, the rats rapidly adapted to this diet. The diarrhea disappeared; new hair appeared; the fur resumed a smooth sleek appearance; and weight increment for the next 2 weeks of feeding approached that of animals fed the basal ration with glucoascorbic acid omitted. In contrast to above, rats fed the basal ration supplemented with 20% alfalfa meal exhibited no retardation in growth. Diarrhea did not occur; they all survived; their fur remained smooth and sleek and they appeared indistinguishable in gross appearance and weight increment from rats fed the basal ration with glucoascorbic acid omitted. Experiments were subsequently conducted to determine comparative effects of graded levels of alfalfa meal, alfalfa fractions, supplements of known nutrients, and other materials of plant and animal origin, on response of immature rats fed a purified ration containing 4% glucoascorbic acid. The animals employed and the procedures were similar to those described above, except that the experimental period was shortened to one week. The shorter period was employed since previous findings had indicated that deleterious effects of glucoascorbic acid administration were most marked during the first 7 days of feeding. One group of rats was fed the basal ration; another received the basal ration with glucoascorbic acid omitted and replaced by an equal amount of sucrose; the remaining groups were fed the basal ration with supplements listed in Table I.

Results. In agreement with previous findings, ingestion of a purified ration containing 4% glucoascorbic acid resulted in significant retardation in growth and occurrence of severe diarrhea in the immature rat. These effects were completely counteracted by concurrent administration of alfalfa meal at 20% level in the diet. Alfalfa meal when fed at 10% level in the diet, also exhibited significant activity although the effects obtained at this level were less pronounced than when

TABLE I. Effects of Alfalfa Meal, Supplements of Known Nutrients and Other Materials of Plant and Animal Origin on Response of Immature Rats Fed Toxic Doses of Glucoascorbic Acid.*

Supplements fed with basal ration	No. of animals	Initial wt (g)	Avg gain in body wt during exp.† (g)	Diarrhea‡
None	12	40.3	7.6 ± 1.5 (9)	+++
2.5% alfalfa meal	"	40.2	10.5 ± 1.7 (12)	+++
5% <i>Idem</i>	"	40.2	12.8 ± 1.6 (12)	++
10% "	"	40.3	18.4 ± 1.7 (12)	+
20% "	"	40.3	23.2 ± .9 (12)	0
5% dried alfalfa juice	"	40.5	7.2 ± 1.2 (11)	+++
15% alfalfa residue	"	40.3	20.9 ± 1.5 (12)	0
2.5% alfalfa ash	6	40.2	9.0 ± 1.2 (6)	+++
20% rye grass	6	40.3	18.2 ± .9 (6)	0
20% orchard grass	6	40.2	21.4 ± 1.3 (6)	0
20% wheat grass	6	40.3	19.9 ± 1.6 (6)	0
20% fescue grass	6	40.3	18.6 ± 1.2 (6)	0
20% oat grass	6	40.1	20.2 ± .8 (6)	0
10% desiccated liver N.F.	6	40.2	5.2 ± 1.6 (5)	+++
10% yeast	6	40.2	11.9 ± 1.3 (6)	++
5% Vigofac	6	40.2	5.4 ± 1.0 (6)	+++
B vitamins, C and K§	6	40.3	.1 ± 1.7 (4)	+++
Vitamins A, D and E	6	40.2	1.5 ± .8 (6)	+++
5% salt mixture	6	40.1	5.4 ± .7 (5)	+++
5% corn oil	6	40.4	4.6 ± .7 (6)	+++
5% cellulose	6	40.2	9.5 ± 2.0 (6)	++
10% casein	6	40.2	8.2 ± 2.3 (5)	+++
Mixed supplements¶	6	40.3	14.0 ± 1.7 (6)	++
2% flavonoids**	6	40.2	6.5 ± .8 (3)	+++
Aureomycin HCl††	6	40.2	.2 ± .6 (4)	+++
Basal ration without gluco- ascorbic acid	12	40.3	26.5 ± 1.8 (12)	0

Values in parentheses indicate No. of animals which survived and on which averages are based.

* Alfalfa samples were kindly provided by Dr. S. Tenkoff of Nutrilite Products, Buena Park, Calif. Dehydrated rye grass, orchard grass, wheat grass, fescue grass and oat grass were obtained from National Chlorophyll & Chemical Co., Lamar, Col. Desiccated liver N. F. was obtained from Wilson Laboratories, Chicago, Ill., yeast (Primary Dried Yeast, Strain No. 200) from Anheuser, Busch, St. Louis, Mo.; cellulose (Solka Floc BW 200) from Brown and Co., Boston, Mass.; salt mixture (Hubbel, Mendel and Wakeman Salt Mixture) and casein (Vit-free Test Casein) from General Biochemicals, Chagrin Falls, O.; and Vigofac from Chas. Pfizer and Co., Brooklyn, N. Y.

† Including stand. error of mean calculated as follows: $\sqrt{\frac{\sum d^2}{n}} / \sqrt{n}$ where "d" is deviation from the mean and "n" is No. of observations. Duration of experimental period was 7 days.

‡ Degree of diarrhea was classified as follows: 0, none; +, mild; ++, intermediate; +++, severe.

§ These vitamins were added per kg of diet at the same level that they were incorporated in the basal ration.

|| 5000 U.S.P. units of vit. A, 500 U.S.P. units of vit. D₂ and 100 mg alpha-tocopherol acetate per kg diet.

¶ 10% casein, 5% cellulose, 5% corn oil, 5% Hubbel, Mendel and Wakeman Salt Mixture, and the vitamin supplements indicated in footnotes § and ||, Table I.

** The following flavonoids were each fed at a 0.4% level in the diet: Calcium flavonate glycoside, naringen, hesperidin complex, hesperidin methyl chalcone and lemon bioflavonoid complex. The flavonoids were obtained from Sunkist Growers, Inc., Ontario, Calif.

†† 100 mg aureomycin HCl/kg diet.

fed at the higher level of supplementation. No significant effects were obtained when alfalfa meal was fed at a 2.5 or 5% level.¶ The active factor (or factors) in alfalfa meal was retained in the alfalfa residue fraction

(the water washed pulp remaining after extraction of the juice). Dried alfalfa juice when fed at a level corresponding to amount present in 20% alfalfa meal was devoid of activity. Dehydrated rye grass, orchard

grass, wheat grass, fescue grass and oat grass when fed at a 20% level, were as active as an equal amount of alfalfa meal. Desiccated liver and yeast when fed at 10% level and a product derived from fermentation solubles[¶] when fed at a 5% level were devoid of activity.^{**} Aureomycin HCl when fed at a level of 100 mg/kg of diet was also without protective effect; on the contrary the supplement of aureomycin HCl appeared to accentuate the toxic effects of glucoascorbic acid administration. Alfalfa ash at a level corresponding to the amount provided by 20% alfalfa meal and assorted flavonoids at a 2% level were also without significant effect.

Discussion. Considerable data are available indicating that alfalfa contains one or more factors, apparently distinct from any of

¶ Weight increment of rats fed basal ration and the basal ration plus alfalfa rations was directly correlated with amount of food ingested. Food consumption was least for rats fed the basal ration and the basal ration plus 2.5% alfalfa meal diet and greatest for rats fed 20% alfalfa meal supplement. Since glucoascorbic acid was incorporated in the diet, however, an increased food intake was associated with a higher intake of glucoascorbic acid. It is apparent, therefore, that the greater growth of rats fed the 20% alfalfa meal supplement occurred despite the fact that these animals ingested the largest amounts of glucoascorbic acid.

¶ Vigofac, Chas. Pfizer and Co., Brooklyn, N. Y.

^{**} Subsequent findings indicate that pectin, agar and 3 different lignin preparations (indulin A, B and C) were also without significant effect when each was fed at 5% level in the diet. A sample of alfalfa meal that was thoroughly extracted with water, then extracted for 15 hours with n-hexane followed by 8 hour extraction with 80% acetone-water solvent showed significant activity when fed at 10% level.

†† The active factor in alfalfa and other succulent plants has tentatively been designated the P.R. (plant residue) factor. One unit has arbitrarily been defined as the minimum amount of material which, when fed daily for 1 week to immature rats on a basal purified ration containing 4% glucoascorbic acid, will promote an average weight increment of 10 g for the 7 day period over that of rats fed basal ration alone. The alfalfa meal employed had approximately 1 unit of P.R. factor activity/g.

the known nutrients, which are essential for optimal growth and physiologic performance under various experimental conditions(3,4). The effects of alfalfa supplementation are particularly marked in animals exposed to certain stressor agents(2,5,6). Present findings indicate that alfalfa and other succulent plants counteracted the toxic effects of massive doses of glucoascorbic acid in the rat. The protective factor^{††} was distinct from any of the known nutrients and was retained in the alfalfa residue fraction (water washed pulp remaining after extraction of the juice). This is the same alfalfa fraction that was active in counteracting the inhibitory effects of alpha-estradiol on ovarian development(6), in prolonging survival of hamsters fed highly purified diets(7), in promoting growth of guinea pigs fed a mineralized dried milk ration(8), and in prolonging survival of rats fed massive doses of desiccated thyroid or iodinated casein(9).

Summary. Ingestion of a purified ration containing 4% glucoascorbic acid resulted in a significant retardation in growth and occurrence of severe diarrhea in the immature rat. These effects were completely counteracted by concurrent administration of dried alfalfa and other succulent plants. The protective factor in alfalfa was retained in the alfalfa residue fraction (water washed pulp remaining after extraction of the juice). Supplements of all known nutrients were without significant effect.

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Received May 3, 1957. P.S.E.B.M., 1957, v95.

Effects of Flavonoids on Response of Rats Fed Massive and Suboptimal Doses of Vit. A.* (23320)

BENJAMIN H. ERSHOFF

Department of Biochemistry and Nutrition, University of S. California, Los Angeles

Prolonged ingestion of massive doses of vit. A results in growth retardation, spontaneous leg fractures, paralysis and other toxic manifestations including death in the immature rat, the severity of symptoms and their rapidity of onset being proportional to dosage of vit. A administered (1-3). A vit. A intake of 10,000 to 12,500 U.S.P. units/day results in a significant retardation in growth; the dosage required to produce more severe symptoms (including leg fractures) in immature rats has generally been found to range from 25,000 to 50,000 U.S.P. units of vit. A/day. In the present communication data are presented indicating that administration of bioflavonoids significantly affects the physiologic response to massive but relatively non-toxic doses of vit. A in the immature rat.

Procedure. The basal flavonoid-free diet employed consisted of sucrose, 66%; casein,[†] 24%; salt mixture,[‡] 5%; and corn oil, 5%. To each kg of above diet were added the following crystalline vitamins: thiamine hydrochloride, 10 mg; riboflavin, 10 mg; pyridoxine hydrochloride, 10 mg; calcium pantothenate, 60 mg; nicotinic acid, 100 mg; ascorbic acid, 200 mg; biotin, 4 mg; folic acid, 10 mg; para-aminobenzoic acid, 400 mg; inositol, 800 mg; vit. B₁₂, 150 µg; 2-methylnaphthoquinone, 5 mg; choline chloride, 2 g; vit. A, 5000 U.S.P. units; vit. D₂, 500 U.S.P. units; and alpha-tocopherol acetate, 100 mg. The vitamins and the various test additives were incorporated in the basal diet in place of an equal amount of sucrose. Diets were made up weekly and stored under refrigeration when not in use. The animals were fed

daily and all food not consumed 24 hours after feeding was discarded. The rats were housed in metal cages with raised screen bottoms (2 or 3 animals/cage) and were provided with food and water *ad libitum*. Feeding was continued for 6 weeks or until death, whichever occurred sooner.

Results. Exp. 1. Two hundred and forty-seven female rats of the Holtzman strain were selected at an average weight of 49.2 g (range 44 to 52 g). Animals were divided into 26 comparable groups. One group was fed the basal diet; the remaining groups were fed the basal diet with supplements listed in Table I. The ingestion of a purified ration containing 3.75 million U.S.P. units of vit. A palmitate/kg of diet resulted in a significant retardation in growth and occurrence of spontaneous leg fractures in the immature rat. These symptoms of hypervitaminosis A were accentuated by the concurrent feeding of flavonoids (calcium flavonate glycoside, naringen, hesperidin complex, hesperidin methyl chalcone, lemon bioflavonoid complex or rutin) at levels of 0.5, 1 or 2% of diet. This was evidenced by the following criteria: (a) weight increment of rats fed the above flavonoids in conjunction with the vit. A supplement was significantly less than that of rats fed the vit. A supplement alone, (b) 90 to 100% of the rats fed both the flavonoid and vit. A supplements developed leg fractures in contrast to an incidence of 39% in animals fed the vit. A supplement alone, and (c) 50 to 100% of rats fed flavonoids in conjunction with the vit. A supplement succumbed during the 6 week experimental period in contrast to 0% mortality in animals fed the vit. A supplement alone. In general the effects of the flavonoids in accentuating symptoms of hypervitaminosis A were as marked when fed at a 0.5% level in the diet as when they were fed at higher levels. When the flavonoids were incorporated at 2% level in the basal diet (without the vit. A supple-

* Communication No. 432 from Department of Biochemistry and Nutrition, Univ. of So. California. This investigation was supported in part by grant-in-aid from Sunkist Growers, Ontario, Calif.

[†] Vitamin-free Test Casein, General Biochemicals, Chagrin Falls, O.

[‡] Hubbel, Mendel and Wakeman Salt Mixtures, General Biochemicals, Chagrin Falls, O.

TABLE I. Effects of Flavonoids on Symptoms of Hypervitaminosis A in the Immature Rat.*

Supplements fed with basal diet	No. of animals	Initial wt (g)	Body wt (g) after following days of feeding†		% of rats with fractures	% surviving	Avg survival time of decedents (days)
			21st	42nd			
None	7	49.0	133 (7)	173 (7)	0	100	
2% calcium flavonate glycoside	7	49.2	115 (7)	160 (7)	0	100	
2% naringen	7	49.2	122 (7)	162 (7)	0	100	
2% hesperidin complex	7	49.2	131 (7)	172 (7)	0	100	
2% hesperidin methyl chalcone	7	49.2	123 (7)	167 (7)	0	100	
2% lemon bioflavonoid complex	7	49.0	132 (7)	167 (7)	0	100	
2% rutin	7	49.2	130 (7)	171 (7)	0	100	
3.75 million units vit. A palmitate/kg of diet	18	49.1	106 (18)	131 (18)	39	100	
<i>Idem</i> + following supplements:							
.5% calcium flavonate glycoside	10	49.2	76 (10)	83 (4)	100	40	31
1 % <i>Idem</i>	10	49.1	73 (10)	61 (2)	100	20	34
2 % "	10	49.0	78 (8)	72 (1)	100	10	30
.5% naringen	10	49.3	68 (7)		100	0	26
1 % "	10	49.4	73 (6)	81 (1)	100	10	25
2 % "	10	49.4	72 (5)	76 (1)	100	10	22
.5% hesperidin complex	10	49.3	77 (6)	84 (2)	90	20	26
1 % <i>Idem</i>	10	49.2	75 (8)	79 (2)	100	20	28
2 % "	10	49.2	78 (9)	81 (5)	100	50	32
.5% hesperidin methyl chalcone	10	49.2	67 (7)		100	0	27
1 % <i>Idem</i>	10	49.2	61 (8)		100	0	27
2 % "	10	49.3	61 (6)		100	0	22
.5% lemon bioflavonoid complex	10	49.3	64 (8)	52 (2)	100	20	25
1 % <i>Idem</i>	10	49.4	61 (8)		100	0	26
2 % "	10	49.3	67 (9)		100	0	25
.5% rutin	10	49.4	57 (7)		100	0	22
1 % "	10	49.4	49 (2)		100	0	19
2 % "	10	49.3	57 (9)		100	0	25

* The calcium flavonate glycoside, naringen, hesperidin complex, hesperidin methyl chalcone and lemon bioflavonoid complex employed were kindly provided by Mr. W. E. Baier of Sunkist Growers, Ontario, Calif. Vit. A supplement was Synthetic Vit. A Palmitate in Corn Oil (1 million U.S.P. units/g), obtained from Hoffmann-La Roche, Nutley, N. J.

† Values in parentheses indicate No. of animals which survived and on which averages are based.

ment), no deleterious effects were observed on any of the rations in respect to weight increment, gross appearance, incidence of fractures or per cent survival.

Exp. 2. In Exp. 1 data were obtained on the effects of flavonoids on the response of rats administered excessive amounts of vit. A. The following experiment was undertaken to determine the effects of flavonoids on vit. A-depleted rats fed suboptimal amounts of this vitamin. The animals employed were female rats of the Univ. of S. California strain in which the dietary regime of mother rats was

such as to make the young rats satisfactory for bioassay studies for vit. A. The mother rats were maintained for at least 2 months previous to breeding and for 7 days after birth of the litter on Sherman diet B(4) without addition of supplementary lettuce or meat. Litters were reduced to 7 at 3 days of age, and on the 7th day mothers and litters were placed on vit. A-free, flavonoid-free diet identical to basal diet employed in Exp. 1 except that the vit. A was omitted from this ration. The young rats were weaned at 21 days of age and were continued on the above

diet until they were depleted of vit. A. The end of depletion period was set as 5th day on which rats on the vit. A-free, flavonoid-free diet showed stationary or decreasing weight. The average time of depletion was 21 days after weaning (range 18 to 23 days); and average body weight at time of depletion was 91 g (range 68 to 115 g). On the day of depletion animals were divided into 8 comparable groups of 10 rats each. Four groups were fed vit. A-free, flavonoid-free diet indicated above supplemented with (1) 0, (2) 100, (3) 250, and (4) 1000 U.S.P. units of vit. A palmitate/kg of diet. The remaining 4 groups were fed diets identical with the 4 rations indicated above but containing in addition 5 g of hesperidin complex and 5 g of lemon bioflavonoid complex/kg of diet. The flavonoids were added in place of an equal amount of sucrose. Animals were placed in metal cages with raised screen bottoms and were fed the above diets *ad libitum* for 28 days or until death, whichever occurred sooner.

All rats fed the vit. A-free diet either with or without the flavonoids succumbed after an average survival of 7 to 8 days (range 4 to 16 days). All rats fed vit. A-free diet supplemented with 100 U.S.P. units of vit. A palmitate/kg of diet either with or without the flavonoids also succumbed within 28 days. Average survival time on the non-flavonoid diet was 14 days, whereas average length of survival on the flavonoid-containing ration was 12 days. At higher levels of vit. A intake, however, the flavonoids exhibited a highly significant effect. Only 2 of the 10 rats fed the non-flavonoid ration supplemented with 250 U.S.P. units of vit. A/kg of ration survived the experimental period of 28 days (average weight increment of the 2 survivors was 29 g). In contrast 8 of the 10 rats fed the flavonoid-containing ration at the same level of vit. A supplementation survived (average weight increment of the 8 survivors was 25 g). Of the rats fed 1000 U.S.P. units of vit. A/kg of ration, 8 of the 10 rats in the non-flavonoid group survived with average weight increment of 47 ± 2.4 g.[§] Of rats fed the same level of vit. A in presence of flavonoids, 9 out of 10 survived with average

weight increment of 69.4 ± 5.0 g.[§] It is apparent from these findings that supplements of hesperidin complex and lemon bioflavonoid complex when fed at 0.5% level in the diet were without significant effect when fed with rations containing 100 U.S.P. units of vit. A or less/kg of ration but significantly increased both the number of animals surviving and the average increment in body weight of vit. A-depleted rats fed diets containing 250 and 1000 U.S.P. units of vit. A respectively/kg of diet. It would seem from these findings that the above flavonoids were active in increasing absorption and/or utilization of vit. A not only when this vitamin was present in excess (Exp. 1) but under conditions of suboptimal intake as well.

Discussion. Present findings indicate that flavonoids can significantly affect the response of immature rats fed purified rations containing either excessive or suboptimal amounts of vit. A. When immature rats were fed flavonoid-free diets containing massive but relatively non-toxic doses of vit. A, supplements of calcium flavonate glycoside, naringen, hesperidin complex, hesperidin methyl chalcone, lemon bioflavonoid complex or rutin resulted in a striking accentuation of the symptoms of hypervitaminosis A. This was evidenced by a more pronounced retardation in growth, a higher incidence of spontaneous leg fractures and a significantly greater mortality in rats fed the flavonoid-containing rations than in those fed similar diets with the flavonoids omitted. These symptoms were identical in appearance, time of onset, severity and incidence to those of rats fed the flavonoid-free diet supplemented with amounts of vit. A considerably in excess of the amounts present in above rations. Supplements of hesperidin complex and lemon bioflavonoid complex also significantly increased both the number of animals surviving and the average increment in body weight of vit. A-depleted rats fed diets containing 250 and 1000 U.S.P. units of vit. A respectively/kg of diet. These find-

[§] Including standard error of mean calculated as follows: $\sqrt{\frac{\sum d^2}{n}} / \sqrt{n}$ where "d" is deviation from the mean and "n" is number of observations.

ings suggest that the above flavonoids may have promoted an increased absorption and/or utilization of vit. A. That unidentified factors exist in natural foodstuffs which promote utilization of vit. A has been reported by a number of investigators. Palm kernel meal(5,6), coconut cake(5,6), acetone-extracted herring roe(5,6), soybean lecithin (7,8), yeast(9), fish solubles(10,11), alfalfa and other succulent plants(12), liver(12) and other substances including the tocopherols(13-15), xanthophyll(16), aureomycin(17,18) and DPPD(19,20) have all been shown to improve vit. A utilization as judged by various indices in experimental animals. Present findings indicate that the flavonoids also have significant activity in this regard and suggest that the activity of at least some of the natural foodstuffs indicated above may have been due, at least in part, to their flavonoid content.

No data are available to indicate the mechanism(s) whereby the flavonoids indicated above exert their physiologic effects. The possibility that the flavonoids are functioning as antioxidants that prevent destruction of vit. A in the diet and hence leave more vitamin present for absorption appears to be ruled out by chemical tests which indicate that no detectable destruction of vit. A occurred in the purified basal diet supplemented with 2.5 million U.S.P. units of vit. A palmitate/kg of ration after 30 days of refrigeration. Under conditions of our experiment, diets were made up at weekly intervals and were consumed within 10 days of the time they were made. In addition no significant difference in response occurred between rats fed the above ration and animals fed an identical diet which was made up daily immediately before feeding. Flavonoids, however, can function not only as *in vitro* antioxidants (21) but in the intact animal as well(22,23). Hence further studies are indicated to determine to what extent the effects of flavonoids in modifying the response to vit. A feeding under conditions of our experiment were due to their function as biological antioxidants which protected vit. A from destruction in the intestinal tract and/or the animals' own tis-

sues. It must be emphasized that the effects of the flavonoids in accentuating symptoms of hypervitaminosis A in the rat occurred with diets containing approximately 2000 to 3000 times the amount of vit. A required for optimal growth, breeding and longevity as determined by Sherman and co-workers(24,25). Preliminary findings indicate that with diets containing smaller amounts of this nutrient (up to 1000 times the optimal vit. A intake) the flavonoids did not accentuate symptoms of hypervitaminosis A. In view of these findings the possibility that flavonoids might induce symptoms of hypervitaminosis A in man even when fed with large therapeutic doses of this nutrient would appear to be extremely remote. On the contrary present findings indicate that flavonoids may play an important role in the treatment of vit. A deficiency states, not only those arising from the ingestion of diets deficient in this vitamin but those resulting from impaired absorption or utilization of this nutrient as well.

Summary. Supplements of calcium flavonate glycoside, naringen, hesperidin complex, hesperidin methyl chalcone, lemon bioflavonoid complex or rutin at levels of 0.5 to 2% of the diet significantly increased the vit. A utilization of immature rats fed massive doses of this vitamin. Flavonoids also increased the apparent utilization of this nutrient in vitamin A-depleted rats fed suboptimal amounts of vit. A.

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Received May 3, 1957. P.S.E.B.M., 1957, v95.

Failure of Hypothalamic Tissue to Reinitiate Thyrotropin Production By Mouse Pituitaries in Tissue Culture.* (23321)

WARNER H. FLORSHEIM, DAVID T. IMAGAWA AND MONTE A. GREER†

U. S. Veterans Hospital, Long Beach Calif. and School of Medicine, University of California at Los Angeles

Guillemin and Rosenberg(1) reported that pituitary explants, which normally lost their ability to produce corticotropin rapidly in tissue culture, could be stimulated to reinitiate corticotropin production by the addition of hypothalamic explants. The stimulating potency resided in the median eminence, a region which previously had been shown to be involved in control of corticotropin production in the intact animal(2).

The importance of hypothalamic centers in regulating production of thyrotropin by the pituitary has been established(3,4,5), but the mechanism of control has not. Greer(3) has postulated the existence of 2 thyrotropins, a "metabolic" and a "growth" factor, and the production of the latter appears to depend upon both pituitary and hypothalamic centers. We therefore thought it of interest to utilize Guillemin and Rosenberg's technic to see whether evidence for a direct stimulation by hypothalamic tissue of hypophyseal thyro-

tropin production could be obtained.

Materials and methods. Pituitaries were taken from adult male Swiss albino mice under sterile conditions. The tissues were washed in Hanks' balanced salt solution and quartered with a sharp scalpel. Four quartered pituitaries were placed in the lower third of 16 x 150 mm test tubes which had been coated with 3 drops of chicken plasma. Following coagulation of the plasma, 1.0 ml of nutrient fluid was added, the tubes stoppered and incubated in a Roller Drum at 36°. Thirty-two such tubes were prepared in this study. The tissues were observed daily for growth and the nutrient medium was changed twice a week. The removed medium was kept at -35° prior to analysis for thyrotropin. Hypothalamic explants were added to 20 of the tubes on the twelfth day of *in vitro* growth and imbedded in a fresh plasma clot. Two entire mouse hypothalami, extending from the optic chiasm to the mammillary bodies were minced into 1 mm³ fragments and added to each tube containing 4 pituitaries. The nutrient fluid consisted of 20% Beef Serum, 10% Chicken Embryo Extract, 20% Hank's

* Aided in part by grants from U. S. Public Health Service.

† Present address: University of Oregon Medical School, Portland

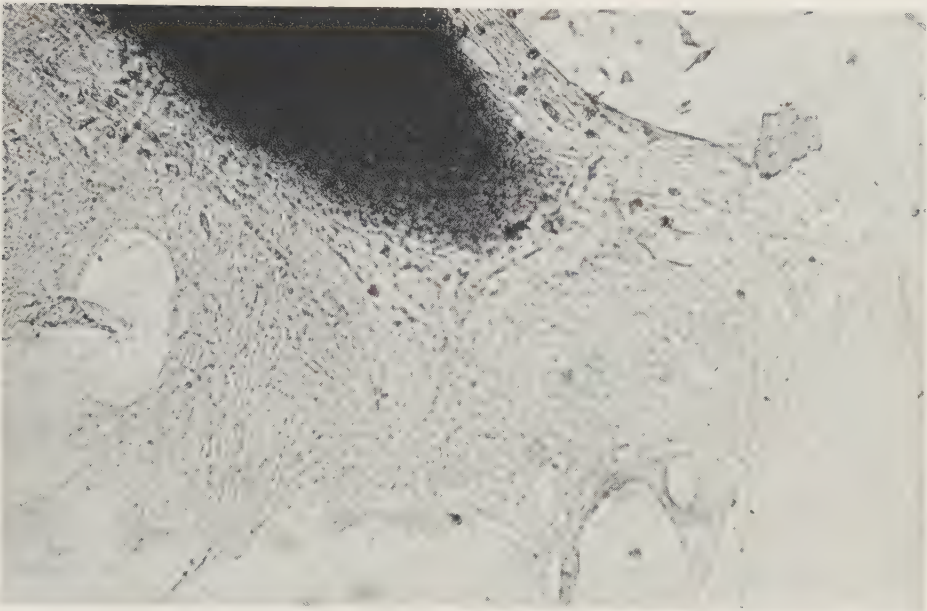


FIG. 1. Photomicrograph of typical pituitary explant after 6 days of *in vitro* growth. Unstained preparation ($\times 100$).

Balanced Salt Solution containing trypsin inhibitor and 50% "199" Solution(6). Phenol Red was used as a pH indicator and each ml of medium contained 500 U of penicillin and 150 μ g of streptomycin. The adequacy of these tissue culture conditions was confirmed by testing some of the incubation fluids for corticotropin content by means of Hodge's assay(7) in young DOCA-treated rats.† Our results were very similar to those published by Guillemin and Rosenberg(1). But, due to the small number of assays performed, increase in corticotropin content of the media after addition of hypothalamic explants, though marked, was significant only at the 5% level of confidence. Thyrotropin analysis was performed by 2 unrelated assays: the assay of Florsheim *et al.*(8) based on *in vitro* stimulation of phospholipid labelling in beef thyroid slices, and a modification of the chick assay of Gilliland and Strudwick(9). The modifications of the latter method consisted of adding 0.15% propylthiouracil(10) and 1% thyroid powder to the diet of the chicks subsequent to the initial 24 hour period allowed for uptake and binding of I^{131} in the

thyroid gland. Ten white Leghorn cockerels were used per sample, each receiving 1.0 ml of test solution in 2 subcutaneous injections.

Results. All pituitary explants grew very well, as shown in Fig. 1, and there was no evidence of bacterial contamination at any time. Hypothalamic tissue grew much more slowly than pituitary, but there appeared to be no interference with the normal growth of both tissues in mixed cultures. Tissues grew for approximately 30 days before becoming granular in appearance. Tissue culture fluid analyses showed a rapidly decreasing rate of thyrotropin output by the pituitary explants. No thyrotropin could be detected in any of the nutrient fluids beyond the second change by either assay. The concentration in the first change of medium averaged about 80 mU per tube, in the second about 20 mU and thereafter no activity was detectable even when injected into chicks at a level of 2 ml per chick. Since the minimum amount of thyrotropin which can be detected reliably by the assay procedures used is 3 mU, it appears that less than 3 mU was liberated into the tissue fluid per 4 pituitaries in 3-4 days. This amount did not increase after addition of hypothalamic explants. Hypothalamus growing

† We are grateful to Dr. R. Gaunt of Ciba Pharmaceutical Products for supplying DOCA.

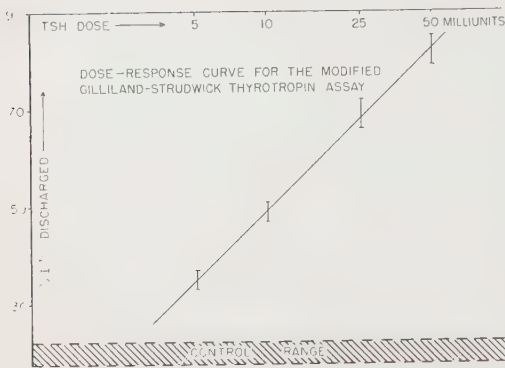


FIG. 2. Dose-response curve for modified Gilliland-Strudwick chick assay showing means and standard errors of response at 4 levels of thyrotropin.

alone in tissue culture also produced no detectable thyrotropin, as was to be expected.

A few of the samples were also analyzed by the chick assay of Greenspan(11) and found to be devoid of activity. However, since only 0.2 ml of the test solution can be injected by the intracardiac route employed in this method, the use of this assay did not extend the detectable limit of thyrotropin concentration. Since the method of Greenspan is less accurate than the other 2 methods, we abandoned its use after only a few assays.

Cultures were checked for growth and thyrotropin production for 25 to 32 days. At the end of this period, the tissue began to appear granular. The remaining tissue and plasma clot were therefore removed from the tubes, homogenized in saline and analyzed for thyrotropin content in chicks. They contained no detectable amounts of the hormone.

It was established by chick assay that a commercial thyrotropin preparation (Parke, Davis "50P4") could be recovered quantitatively after being in contact for one week with growing explants of pituitary and hypothalamus. We also showed that less than 10% of pituitary thyrotropin content is lost from the gland during the explanting procedures.

The chick assay for thyrotropin as used in our laboratory was useful in the range of 3 to 50 mU of USP thyrotropin reference substance (Fig. 2). The Index of Precision (λ), calculated by Emmens' method(12) was 0.23 and was very reproducible with chicks ob-

tained from the same source. The sensitivity and precision of the chick assay were very similar to those of the thyroid slice assay employed.

Discussion. With the 2 assay procedures employed we were unable to detect the production of more thyrotropin by mouse pituitary explants than that originally present in the glands. The elution of this material occurred during the first 2 changes of the incubation medium, and we recovered approximately 25 mU per gland, which is in fair agreement with the values for mouse pituitary thyrotropin content given by Bates(10). Since the growing tissue did not metabolize exogenous thyrotropin and since the entire hormone content of the original gland was recovered during the first week of incubation, it is unlikely that newly formed thyrotropin was lost to analysis by metabolic degradation. The thyrotropin produced, if any, during a week of growth in tissue culture thus was less than 4% of the original content of the glands, even after addition of hypothalamus explants.

The assays employed were chosen to reflect different aspects of thyrotropin action. The Gilliland-Strudwick assay is clearly a measure of "metabolic" thyrotropin, since it measures the discharge of labelled thyroid hormone from the target gland. The assay based on the phospholipid labelling in surviving beef thyroid slices was chosen because it proved completely unresponsive to all goitrogens including propylthiouracil, thiocyanate and iodide, and thus seemed to measure an aspect of thyrotropin action unrelated to the effects ascribed to the metabolic factor. However, no new synthesis of any thyrotropin could be detected by this method. It appears, therefore, that intervention of the hypothalamus in thyrotropin synthesis is not as direct as it has been shown to be for corticotropin production, and no hypothalamic neurohumor could be demonstrated by the technics used in this study.

Summary. Contrary to Guillemin's findings regarding the reinitiation of corticotropin production by pituitary explants in the presence of hypothalamic tissue, no thyrotropin

was produced by mouse pituitary explants even after addition of growing hypothalamic tissue. Since the assay procedures employed would have detected 3 mU of thyrotropin, less than 4% of the original thyrotropin content of the explants was produced per week.

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Received May 6, 1957. P.S.E.B.M., 1957, v95.

Alteration of Hepatic Storage of Radiolabeled Vit. B₁₂* (23322)

ALFRED DOSCHERHOLMEN AND PAUL S. HAGEN

(With technical assistance of Margaret Liu)

*Medical Service, Veterans Administration Hospital and Department of Medicine,
University of Minnesota Medical School, Minneapolis*

The liver is the most important storage organ for vit. B₁₂. After oral or parenteral administration of radiocobaltlabeled cyanocobalamin, surface counting over various sites has shown that the liver accumulates most of the radioactivity(1-4). The liver also retains the radioactivity with great avidity. Indeed, from 86 to 94% of the maximal activity was found after 3 months(1), and 50% after one year(5). Moreover, the plateaus of liver radioactivity in 4 control subjects were unaffected by single, intramuscular doses of 1 mg of "cold" vit. B₁₂ given on the seventh day (6).

The objective of the present investigation was to observe the effect of a large series of 1 mg parenteral doses of "cold" vit. B₁₂ on hepatic storage of radioactivity after orally administered radiolabeled cyanocobalamin.

Materials and methods. The Co⁶⁰ labeled vit. B₁₂ used in these experiments had a specific activity of 1082 μ c per mg. A test dose

of 0.46 μ g vitamin containing 0.5 μ c Co⁶⁰ was administered to 2 healthy female volunteers after an overnight fast in approximately 100 ml of water and breakfast was withheld for 2 hours. Plasma radioactivity(7) as well as a 24 hour urinary excretion study(8) started at 24 hours showed that they both absorbed the vitamin normally. External monitoring over the organ areas was carried out according to the method of Glass(1) employing a NaI (thallium activated) probe scintillation counter with a crystal 1½ inches in diameter and 1 inch thick and with an aperture of 2½ inches in diameter. Uptake of radioactivity over the liver, spleen and precordium was determined 9-13 days after administration of the test dose and at intervals up to 7½ months after the tests. The radioactivities recorded between one and 2 weeks after the tests were taken as the 100% values. One of the subjects was given a series of one hundred 1 mg parenteral injections of nonradioactive cyanocobalamin over the ensuing 7 months, while the other subject served as a control. Twenty-four hour urine collections were made

* This investigation was supported by grant from Eli Lilly and Co. to the University of Minnesota.

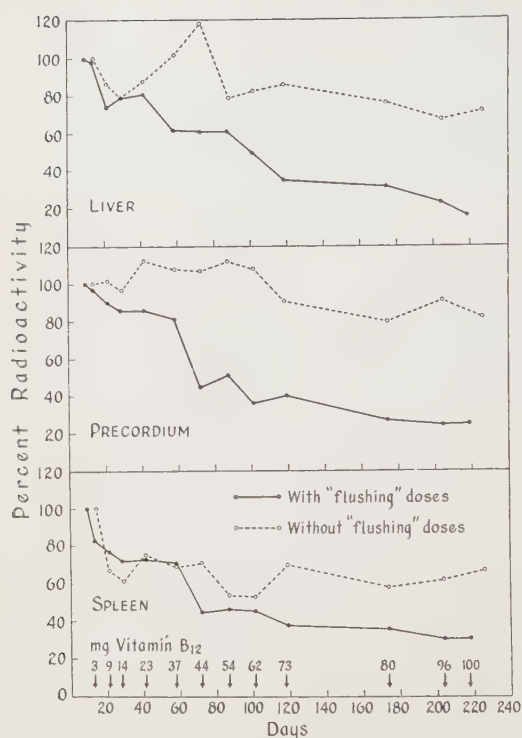


FIG. 1. Effect of parental injections of vit. B₁₂ on body stores of radioactive cyanocobalamin. Net counts/min. for 100% values were 905, 599, and 421 for the liver, precordium and spleen, respectively, in the exp. subject. Comparable figures for control subject were 507, 382, and 295. Cumulative amounts of parenteral "cold" vit. B₁₂ are shown at bottom of graph.

at intervals and the amounts of radioactivity excreted were determined according to a previously described technic(7).

Results. Fig. 1 shows that repeated 1 mg parenteral doses of "cold" vit. B₁₂ caused a marked increase in the rate of disappearance of radioactivity over the liver as well as over the precordium and spleen. The radioactivity over the liver declined to 49.5% 101 days after administration of the test when 62 mg cyanocobalamin had been administered, and to 15.8% 218 days after the test when 100 mg had been given. In the control subject liver radioactivity amounted to 82.6% and 71.4% on the 101st day and the 226th day after the test.

Table I summarizes the amounts of urinary radioactivity in each subject. The control subject did not excrete measurable radioactivity 10 days after the single injection of

TABLE I. Effect of Repeated Intramuscular Injections of "Cold" Vit. B₁₂ on Urinary Excretion of Radioactivity.

Urinary collection started at:	24 hr urinary radioactivity, % of oral test dose	
	Exp. subject	Control subject
24 hr	17.7*	4.9*
8 days	0 †	
9 "	.7*	
10 "		0 †
21 "	.5*	
30 "	.4*	
186 "	.3*	

* Vit. B₁₂ parenterally immediately before start of urinary collection.

† No vit. B₁₂ parenterally.

cyanocobalamin which she received. After the parenteral vit. B₁₂ injections the experimental subject had definite radioactivity in the urine each time it was examined. The amount excreted was 0.7% of the administered dose at the start of the injections and decreased to 0.3% toward the end.

Discussion. The results of this study show that the radioactivity stored in the liver can be reduced by a series of parenteral injections of vit. B₁₂. Because of a simultaneous decline in the radioactivity over the spleen and the precordium, it was thought unlikely that the loss of hepatic radioactivity was due to an accumulation of radioactivity in other organs. The finding of radioactivity in the urine showed that the kidneys were at least one route of loss from the body. A rough estimation indicated that about 40% of the test dose was excreted in the urine during the series of injections which were started the ninth day after administration of the oral test. No attempts were made to prove that the radioactivity was lost in the form of cyanocobalamin, although this has been contended for the urinary radioactivity in the usual Schilling test(9). The findings in this study are in agreement with Schloesser and Schilling's report of a break in the disappearance curve for hepatic radioactivity effected by a few parenteral injections of vit. B₁₂(5).

Although the hepatic radioactivity in the control subject declined slower than in the experimental subject, the rate was still faster than expected from the physical decay of Co⁶⁰ alone. This is in agreement with previous ob-

servations(1,5), and indicates a release of stored radioactivity. Assuming that the stored radioactivity represents radioactive cyanocobalamin(4) and with a normal intake and absorption of vit. B₁₂ this finding suggests an exchange between stored cyanocobalamin in the liver and the rest of the body. Such turnover can be enhanced by parenteral injections of large amounts of vit. B₁₂. The loss of the vitamin in the urine can be explained by the assumption that all available binding capacity was exceeded whereby the free cyanocobalamin was excreted by the kidneys.

Summary. Radioactivity measurements were made over the liver, spleen and precordium for 7½ months after oral administration of test doses of radioactive vit. B₁₂. One subject was given a large series of parenteral injections of nonradioactive vit. B₁₂, while another subject served as a control. The disappearance rate of radioactivity was much faster with the repeated parenteral injections. Radioactivity was lost in the urine. These

observations showed that massive parenteral doses of vit. B₁₂ exchanged with cyanocobalamin stored in the liver.

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Received May 6, 1957. P.S.E.B.M., 1957, v95.

Preparation and Some Properties of *Neisseria gonorrhoeae* Endotoxin.* (23323)

HENRY TAUBER AND WARFIELD GARSON

(With technical assistance of C. S. Adams and D. L. Johnson)

*Venereal Disease Experimental Laboratory, U.S.P.H.S., School of Public Health,
University of N. Carolina, Chapel Hill.*

In the present paper a rapid and simple method for the preparation of a stable fraction containing dry, soluble endotoxin from dry gonococcus cells is described. This material offers a fairly toxic product suitable for *in vivo* and *in vitro* studies. It has been observed that washed gonococcus cells dried over sodium hydroxide *in vacuo* dissolve readily at pH 13. Under these conditions presumably all of the cell-bound endotoxin is freed. Other investigators(1,2) have previously used a much less alkaline pH for the slow extraction of cell-bound endotoxin. Un-

der those conditions only a small portion of the endotoxin is obtained. The gonococcus in these studies was grown in an atmosphere of air using a modified liquid medium which was found to be suitable for the large scale cultivation of the organism.

Materials and methods. *Culturing methods.* Six different strains of gonococcus were used in this study. Five were isolated from patients by Dr. James D. Thayer of this laboratory, one strain was obtained from the American Type Culture Collection (No. 9827). The strains were maintained on dextrose starch agar (Difco) supplemented with 0.1% yeast extract and 1% nutrient agar

* Presented before the Am. Assn. of Immunologists, Chicago, Ill., Apr., 1957.

(Difco) (3). Serial transfers were made every 3 days. From the slants transfers were made to test tubes containing 6 ml portions of liquid medium (described in the procedure to follow). From the test tube cultures which were maintained as a permanent stock, transfers were made to 250 ml Erlenmeyer flasks containing 75 ml portions of liquid medium. These "seeding" cultures and the slants were grown in an atmosphere of CO₂ (candle method). All cultures were grown for 3 days at 36°C. *Large scale cultivation of gonococcus.* The medium had the following composition (g/l): soluble starch, 0.5; yeast extract (BBL, Difco), 1; Bacto nutrient broth, 9; Difco proteose peptone No. 3, 4; dextrose 2; Na₂HPO₄ 3; NaCl 5. The pH after autoclaving was 7.2. Low form Pyrex flasks (2.5 l) containing 1.0 l of medium were employed. Final inoculation was made with 20 ml of seeding culture/l. In this medium the organism grew as well in an atmosphere of air as in one of CO₂. After 6 days of growth at 36° the cells were collected in the Sharples centrifuge. They were washed twice with 0.85% sodium chloride solution and once with distilled water. After each washing the cells were collected in the Servall angle centrifuge at 7000 g. The washed cells were dried over sodium hydroxide *in vacuo*. The yield of dry cells was 60 to 100 mg per l of medium, depending on the strain employed. The American Type Culture Collection strain grew best, but its endotoxin producing capacity was low. 25 l of culture were processed at one time. After completion of this paper we found that the nutrient broth may be omitted provided 0.8% proteose peptone No. 3 is used. Then good growth is obtained in 3 days. In the following experiments a single strain (isolated from a patient) was employed. *Preparation of endotoxin.* 500 mg of dry gonococcus cells were ground in a mortar and the resulting fine powder was then dispersed and lysed with two 6 ml portions of 0.05 N sodium hydroxide (pH 13). The resulting mixture was placed in a 125 ml glass-stoppered Erlenmeyer flask. The mortar and pestle were rinsed with 60 ml of 0.05 N sodium hydroxide and the washings added to the lysate. The flask was stoppered and the mixture shaken for ½ minute.

The mixture was then adjusted to pH 7.4 with 0.5 N acetic acid. If a precipitate of proteins formed it was redissolved by shaking. Cell debris were removed by centrifuging in a cold room in the Servall angle centrifuge at 6600 g. The supernatant was placed in cellophane bags and dialyzed for 20 hours at 4° against 14 l of distilled water. The latter was stirred continuously. A small quantity of sodium chloride was added to the dialyzed supernatant and the endotoxin was precipitated, together with nucleoproteins and small quantities of other proteins, by the addition of 3 volumes of cold acetone. The precipitate was removed by centrifugation and dried over sodium hydroxide *in vacuo*. The yield of dry material was 40% on the basis of dry gonococcus cells. This preparation, which consisted mainly of nucleoproteins, contained 14.24% nitrogen and 1.08% phosphorus. This product was dissolved in a small volume of 0.05 N sodium hydroxide. The resulting solution was adjusted to pH 7.40 with 0.25 N acetic acid, diluted to the desired concentration with sterile distilled water and administered intraperitoneally. It had a MLD (minimum lethal dose) of 2 mg/20-22 g mouse. The animals were observed over a 5-day period.

Results. Effect of trypsin on endotoxin. From 1 g of dry gonococcus cells a cell-free endotoxin-containing solution was prepared as described in the preceding paragraph, except that the solution was not dialyzed. It was divided into 2 equal portions (21 ml each). To one portion 5 ml crystalline trypsin (Worthington, 2x crystallized) solution containing 50 mg of trypsin protein was added, and the mixture adjusted to pH 8.6. To the second portion 5 ml of distilled water was added as a control. To each sample 0.25 ml of 2% merthiolate solution was added. The samples were kept at 36° for 7 days and the pH was maintained at 8.6 by the addition of 0.05 N sodium hydroxide. At the end of the digestion period the samples were dialyzed for 24 hours against distilled water. The MLD of the trypsin-digested sample was 1 mg, that of the control was 2 mg, on dry weight basis. In another experiment an endotoxin-containing solution was kept at pH 7.0. After

3 days of tryptic hydrolysis, most of the endotoxin was destroyed. There remained only a small quantity of toxic nucleoprotein.

Yield, toxicity and properties of different gonococcus fractions. MLD values are close approximates because only a small number of mice were used for each quantity of endotoxin. The dry gonococcus cells had an MLD of 5 mg. The lysate after dialysis showed a removal of 48% of soluble (mostly nucleoprotein) material from the cells. On this basis the MLD was 0.5-2 mg. The acetone precipitate's MLD was 2 mg and the yield was 40% on dry cell basis. An acetic acid precipitate was obtained by precipitating the proteins from the dialyzed lysate at pH 4.2, dissolving the precipitate in dilute sodium hydroxide and lyophilizing the neutralized solution. The MLD of such products made at different times from the same cell source varied from 6 to 30 mg and the yield was 40 to 46%. The tryptic digest which was conducted at pH 8.6 for 7 days contained after dialysis only 6% solids and had an MLD of 1 mg. The control had a solid content, after dialysis, of 16% and an MLD value of 2 mg. An aliquot of the control before dialysis had a solid content of 60% and an MLD of 7 mg. Thus, part of the loss appears to be due to tryptic hydrolysis and part to the alkaline pH. In another experiment without trypsin at pH 13 all of the endotoxin was destroyed in 7 days at 36°. This endotoxin, however, is quite resistant to heat. There was no loss of toxicity when a solution containing 3 mg of endotoxin per ml at pH 7.0 was kept at 60° for 60 minutes.

Deoxynucleic acid content of different gonococcus fractions. Because of the importance of deoxynucleic acid in endotoxin formation and because the results may aid in the elucidation of the chemical nature of this endotoxin, DNA assays were made on most gonococcus fractions. Dry gonococcus cells contained 5.8% DNA. The acetone precipitate's DNA content varied in different preparations between 5.6-6.0%. The tryptic digest, after dialysis, contained 14.0% DNA. The lysate at pH 8.6 kept for 7 days at 36° without trypsin contained 2.4% DNA before dialysis. An aliquot of this lysate, after

dialysis and concentration to the original solid content contained only a trace of DNA. This indicates that the residual portion became small enough to pass through the cellophane membrane. Here the solid content dropped from 60% to 16%. Tryptic hydrolysis resulted in a different breakdown, because after dialysis, both the MLD value and the DNA value were high. Nevertheless much of the endotoxin was destroyed as indicated by the yield on a solid basis.

For DNA analysis the various fractions were hydrolyzed for 15 minutes with boiling 5% trichloroacetic acid in test tubes covered with appropriate glass bulbs to prevent evaporation. Aliquots of the filtrates were analyzed by the diphenylamine method(5), using the Klett-Summerson photoelectric colorimeter with orange filter No. 721. Thymus DNA, very kindly furnished by Dr. J. L. Irvin, was employed for the preparation of a calibration curve.

Discussion. Tryptic hydrolysis (at pH 8.6) followed by dialysis resulted in the removal of much inert material and a 2-fold increase in toxicity. There is, however, considerable loss in endotoxin mostly because of the action of trypsin. Gonococcus endotoxin is quite sensitive to continued exposure to an alkaline pH but quite resistant to heat at pH 7.0. A comparative study of different endotoxin-containing fractions has been made. Our findings indicate that this endotoxin is a protein. There is another kind, a so-called Boivin-type of endotoxin present in certain Gram negative bacteria which has been named "glucolipid." That endotoxin has been found to contain a polysaccharide combined with phospholipid and is prepared by the extraction of bacterial cells with trichloroacetic acid. A small quantity (1.5 mg per g of bacterial protein) of glucolipid is also present in the gonococcus(5).

Summary. 1. An improved method for large scale cultivation of *N. gonorrhoeae* in an atmosphere of air has been described. 2. A rapid method for the preparation of a stable fraction containing dry, soluble gonococcus endotoxin and some of the properties of this product have been presented. This endotoxin appears to be a protein. 3. DNA equivalents

in different gonococcus fractions do not parallel toxicity.

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Received May 7, 1957. P.S.E.B.M., 1957, v95.

Cholinesterases in Human and Ruminant Central Nervous Tissue.* (23324)

JOSEPH BERNSOHN AND LEROY POSSLEY (Introduced by E. Kaplan)

Veterans Admin. Hospital, Hines, Ill., and Department of Neurology and Psychiatry, Northwestern University Medical School, Chicago, Ill.

In comparison to acetylcholinesterase (AChE), the role of butyrylcholinesterase (BuChE) in central nervous tissue has been relatively unexplored. This seems to be due to (a) lack of knowledge of the physiological substrate for BuChE, (b) presence of this enzyme in the glial tissue rather than in the axoplasm(1) which would rule out any function for this enzyme in nerve impulse propagation, and (c) its apparent absence from ruminant central nervous tissue, making it difficult to assess the fundamental function of the enzyme. Its reported absence from ruminant tissue has been based on the observation of Mendel, Mundell and Rudney(2), that benzoyl choline is a specific substrate for BuChE, and no benzoyl choline hydrolysis is evident when tissues from this species are assayed with this substrate(3). Recently, Hardwick(4) reported isolation of an enzyme from bovine plasma that possessed properties of a BuChE, except for its inability to hydrolyze benzoyl choline. This investigation was undertaken to determine whether a BuChE could be demonstrated to be present in bovine brain tissue. If this could be established it might be possible to view the role of this enzyme in brain tissue in a new perspective.

Methods. Because of observations that BuChE activity is higher in the white matter of central nervous tissue than in the gray(5), this part of the brain was used for assay. The brains were kept frozen at -15°C until ready

for use, and then allowed to thaw, at which time the desired samples were dissected out, a 1:5 homogenate in 0.023 M NaHCO_3 -0.154 M NaCl prepared, and aliquots ranging between 0.5 and 1 ml were used in the determinations. Activity was measured in the rotary Warburg apparatus by a modification of the method of Ammon(6), and rates were calculated from the initial velocity, although in most cases the velocities simulated a zero-order reaction during the test period which was usually 60 minutes. Differentiation of the 2 cholinesterases was made possible by the use of a specific acetylcholinesterase inhibitor, mytelase[†] (N' , N' -bis-[2-diethylamino ethyl] oxamide bis-2-chlorobenzyl chloride)(7). Reaction mixtures with the final concentration of reactants in the vessel are given in the tables. In all cases the substrate was tipped in from the side arm after 5 minutes equilibration. All results are the averages of 4 experiments.

Results. The Q values, expressed as μmols of substrate hydrolyzed/g tissue/hr, for human, bovine and sheep central nervous tissue white matter, are shown in Table I. With acetylcholine as a substrate, hydrolysis occurs in the 3 species to varying extents, with human white matter having the highest activity, and bovine white matter the least. To assess the contribution of each of the 2 enzymes, the tissue was then assayed in the presence of 5×10^{-7} M mytelase. It can be

* This work was aided by grant from Nat. Inst. of Neurological Diseases and Blindness, N.I.H.

[†] We are indebted to Winthrop Laboratories for the generous gift of this compound.

TABLE I. Cholinesterases in White Matter of Human, Bovine and Sheep Brain.

Substrate	Human		Bovine		Sheep	
	Q*	% inhibition	Q	% inhibition	Q	% inhibition
AcCh · I, 1×10^{-2} M	24.2		11.7		16.5	
" + inhibitor†	13.3	45.0	3.65	68.8	5.35	67.6
BuCh · Cl, 1×10^{-2} M	33.4		8.7		16.5	
" + inhibitor†	32.2	3.6	8.1	7.0	15.7	5.0
BzCh · Cl, 1×10^{-2} M	8.6		.0		.0	

* Q = μ M substrate hydrolyzed/g wet tissue/hr.† Mytelase, 5×10^{-7} M.Reaction mixture: Substrate, 1×10^{-2} M; buffer, 0.023 M NaHCO_3 -0.154 M NaCl, pH 7.4; atm., 95% N_2 -5% CO_2 ; homogenate equivalent to 200 mg wet tissue; total vol, 2 ml.

seen that the degree of inhibition for human, bovine and sheep white matter was 45.0%, 68.8% and 67.6% respectively. The residual activity for bovine brain was about 27% of the human, and in the case of the sheep brain was 40% of the human value. It can be seen that at this substrate concentration, somewhat less than half of the human enzymatic activity is due to AChE, while in the ruminant tissue, about $\frac{2}{3}$ of the activity is due to this enzyme.

Using butyrylcholine as a substrate, which at the concentration of AChE, here encountered, is a specific BuChE substrate, an appreciable hydrolysis by the brain white matter from the 3 species is observed, which is only slightly inhibited by mytelase, although the activities in the ruminant tissues are lower than in human tissue. The ratios of the inhibited butyrylcholine:inhibited acetylcholine values for the human, bovine and sheep tissues are 2.2, 2.4 and 2.9 respectively, which is in agreement with the observed ratios of activity for these 2 substrates in serum, where the BuChE occurs(8). When benzoylcholine is used as a substrate, only the human

white matter exhibits any activity for this substrate, whereas the ruminant tissue does not hydrolyze this compound.

In Fig. 1, where the % inhibition is plotted against pI (negative of the logarithm of the inhibitor concentration), are shown the action of mytelase on human and bovine grey and white matter. In the basal ganglia, where AChE predominates, the inhibitor is completely effective in inhibiting hydrolysis of acetylcholine above a concentration of 1×10^{-7} M, with a pI_{50} † of 8.2. In white matter, using butyrylcholine as a substrate no inhibition occurs at a concentration of the inhibitor lower than 1×10^{-6} M, and a pI_{50} of 4.4 is observed. This difference in inhibitory activity of 1:6300 to achieve separation in activities between the 2 enzymes makes this compound one of the most satisfactory anticholinesterase agents used for this purpose. Thus the data on inhibition previously obtained on serum and erythrocytes is applicable to central nervous tissue, and validates its use in separating the two enzymes in brain. Double inhibitor experiments using mytelase and 5×10^{-5} M eserine, abolished all enzymatic activity for acetyl- and butyrylcholine.

Discussion. It can be readily observed that there is present in ruminant white matter from the central nervous system, an enzyme which possesses all of the properties of a BuChE, except for its inability to hydrolyze benzoylcholine. It has a greater activity toward butyrylcholine than acetylcholine, and

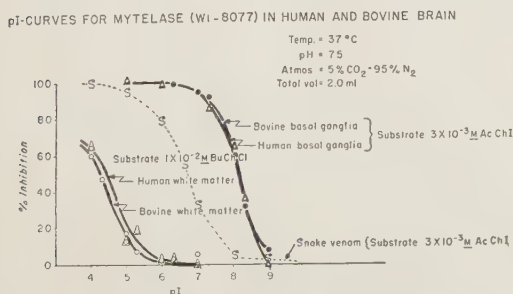


FIG. 1. Effect of mytelase (Wi-8077) on cholinesterases in human and bovine brain.

† pI_{50} = Negative of logarithm of inhibitor concentration required to produce 50% inhibition of the enzymatic system.

the ratio of the activities toward these 2 substrates is similar to the enzyme in human serum. In addition, its activity is completely inhibited by eserine. Other studies(9) have shown that the substrate optimum characteristics are similar to the BuChE in serum, showing increasing activity with higher substrate concentration. All of these observations leave little room for doubt that the enzyme being measured in ruminant brain tissue is the characteristic BuChE found in nervous tissue from non-ruminant species. That this enzyme from ruminant tissue is incapable of hydrolyzing benzoylcholine in contradistinction to the enzyme from non-ruminant sources is of interest and this differential characteristic may lead to studies on the nature of the binding sites on the 2 enzymes which cause such differences in substrate specificity.

If this enzyme is found to be present in other species where it has hitherto been presumed to be absent, then its ubiquity in nervous tissue would imply that it may serve some basic function in metabolism in that organ.

Finally, it must be stated that the use of the terms acetylcholinesterase and butyrylcholinesterase is occasioned not by any conviction as to nomenclature, but rather that the terms hitherto employed have outlived their usefulness, and that some standardization in terminology is required. In this work,

the specificity of each enzyme for chain-length optimum has been used as a basis for identification.

Summary. Studies on the cholinesterase in white matter of human, bovine, and sheep brain indicate that there is present in ruminant white matter a butyrylcholinesterase with properties similar to enzyme in human tissue, except for its inability to hydrolyze benzoylcholine. However, the enzyme from these 2 sources gives ratios of hydrolysis for butyrylcholine : acetylcholine which are alike, and the activity is completely inhibited by eserine. Thus it would seem justifiable to attribute to the enzyme a fundamental role in nervous tissue function, if its presence in other species can be confirmed.

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Received May 7, 1957. P.S.E.B.M., 1957, v95.

Influence of Cortisone and "Stress" upon Persistence and Multiplication of MEF₁ Virus in Hamsters.* (23325)

CONSTANTIN V. TEODORU†

Department of Microbiology, Mount Sinai Hospital, N. Y. City

It has been shown that under the influence of stress, the incidence of paralytic poliomyelitis in experimentally infected hamsters depends on endocrine reactivity to stress of individual animals(1). However, animals re-

acting to stress did not develop poliomyelitis when inoculated intraperitoneally unless proper inoculatory timing provided active virus during a specific stage of their endocrine stress-reaction(1). Observance of such timing requirements appeared to be particularly important for extraneural routes such as intraperitoneal, intramuscular, subcutaneous, etc., where the virus disappears within 3 days

* Aided by grant from Natl. Fn. for Infantile Paralysis.

† Present address: Isaac Albert Research Institute of Jewish Chronic Disease Hospital, Brooklyn, N. Y.

TABLE I. Persistence and Multiplication of MEF₁ Virus Introduced into Interscapular Brown Fat of Hamsters Treated with Cortisone or Submitted to Stress as Compared to Control.

Group	24 hr	Viral titer of ISB* as tested in mice				
		48 hr	72 hr	96 hr	120 hr	144 hr
Cortisone treated	3.4	4.5	5.4	5.9	†	†
Control animals	2.8	3.7	2.8	0	0	0
Sterile intraspinal inj.	2.8	3.8	3.4	1.4	0	0
Fixed on stretcher 6 hr	2	3.6	2.9	2.6	0	2.0
Refrigerated at 4°C 6 days	2.4	3.9	2.0	3.0	2.3	2.4

* Titers are expressed as negative log of the dilution producing a mortality of 50% in mice.

† Not determined.

following inoculation(1). In intracerebrally inoculated animals, where the virus persists 7-10 days, the chance of meeting the endocrine condition favorable for viral progression is considerably greater and, therefore, the importance of experimental timing is significantly reduced(1). Since the cerebral trauma involved in intracerebral inoculation was found to be a stress producing adrenal hypertrophy(1), it was thought that the long persistence of the virus following this route of inoculation may be partly a result of the accompanying stress. This study investigates the influence of various types of stress upon persistence and multiplication of MEF₁ poliomyelitis virus introduced into hamster interscapular brown fat, an extraneural tissue shown to be a site of poliomyelitis virus propagation(4). Normal and cortisone treated animals were used as parallel controls.

Materials and methods. Male Golden Hamsters 25-35 g were used. The interscapular brown fat (ISB) was surgically exposed and 0.1 ml of MEF₁ virus suspension of known infectivity titer was injected into it directly. At varying periods after inoculation the ISB was removed from groups of 3-6 animals, pooled, emulsified and its infectivity titrated in mice. Parallel series of stressed, cortisone-treated and normal hamsters were simultaneously prepared as above. Refrigeration, immobilization for 7 hours on a stretcher, and a pathogen-free intraspinal injection were used as stressing agents. Cortisone, 5 mg, was injected intramuscularly the same day as viral inoculation in the cortisone-treated groups. The viral titers shown in the Table represent the negative log of the dilution which when injected intracerebrally in 0.03 ml amounts in mice produced a 50% mortal-

ity. The LD₅₀ dilution was calculated according to the method of Reed and Muench (2).

Results. Influence of cortisone: Brown fat of cortisone treated hamsters, removed at varying intervals after direct viral inoculation, showed significantly higher infectivity titers in mice than the brown fat of parallel controls (Table I). To compensate for possible presence of cortisone in the brown fat of cortisone-treated animals, the infected brown fat of controls was pooled with equal amounts of brown fat from non-infected but cortisone-treated hamsters, while the infected brown fat of the cortisone-treated group was pooled with non-infected brown fat taken from normal animals. The two resulting emulsions were diluted 1:500 in saline, then inoculated intracerebrally in 2 series of mice (Table II).

On the assumption that the pooling operation had eliminated possible differences in cortisone content, it was concluded that the difference in infectivity of the 2 emulsions demonstrated in Table II was due to a difference in viral concentration. Both Tables I and II indicate that, in addition to enhanced viral multiplication, viral persistence is significantly longer in cortisone-treated than in control animals.

Influence of stress: Table I shows that, when introduced into the brown fat of normal hamsters, MEF₁ virus progression follows a definite pattern of active multiplication during the first 48 hours followed by drop of infectivity titer and disappearance of the virus during the next 48 hours. In the brown fat of hamsters undergoing stress, viral multiplication follows the same pattern as in control animals for the initial 72 hours following inoculation. Beyond that time, however, in

TABLE II. Comparative Infectivity in Mice of Brown Fat Removed from Cortisone Treated and from Control Hamsters at Varying Intervals after Direct Inoculation with MEF₁ Virus.*

Group	Infectivity of brown fat as tested intracerebr. in mice								
	48 hr			96 hr			7 days		
	No. mice	% mortality	"P"	No. mice	% mortality	"P"	No. mice	% mortality	"P"
Emulsion "A"	50	94	0.0001	100	97	0†	100	12	0.01
Emulsion "B"	50	62		50	4		50	0	

* To compensate for possible presence of cortisone exclusively in the cortisone treated group the following tissues were pooled and emulsified: *Emulsion "A"*—50% infected brown fat from cortisone treated hamsters + 50% normal brown fat from non-infected hamsters; *Emulsion "B"*—50% infected brown fat from control hamsters + 50% non-infected brown fat from cortisone treated hamsters.

† Statistically highly significant.

contrast to the control where no virus could be detected, a significant viral titer was demonstrable in the brown fat of hamsters submitted to stress, indicating a longer viral persistence in the latter as compared to the former (Table I).

Discussion. From the above experiments it may be concluded that (a) cortisone or stress delayed significantly the disappearance of MEF₁ virus inoculated into the interscapular brown fat of hamsters, and that (b) in contrast with cortisone, which produced a prompt and significant enhancement of viral multiplication, stress did not induce any deviation from the multiplication pattern exhibited by controls during the initial 72 hours following viral inoculation. This difference in action upon viral multiplication seems to be responsible for the fact that although both cortisone and stress enhanced the total paralytic incidence, the former shortened (3) while the latter prolonged (1) the mean incubation period as compared to controls. Thus, shortening of the incubation period was closely associated with enhancement by cortisone of viral multiplication rate which accelerated the development of poliomyelitis in all susceptible animals. Stress, failing to enhance viral multiplication did not accelerate emergence of disease but, seemingly in consequence of its delaying action upon the disappearance of the inoculated virus, increased paralytic incidence by increasing frequency of belated paralysis (1). The increased number of belated paralytic cases increases the mean incubation time of the group as has been demonstrated previously (1).

Experiments in progress strongly suggest that the above difference between the action of cortisone and that of stress is a function of timing. Actually, in stress, although the end result is cortisone hypersecretion, the latter is preceded by pituitary ACTH discharge, which in recent, unpublished experiments, appears to exert as a side effect an action opposite to cortisone upon the metabolism of brown fat in hamsters. Endogenous ACTH has the same effect as the exogenous introduction, as suggested by metabolic alterations found in the brown fat of hamsters during pregnancy or during the early stage following stress. On the other hand, cortisone-like alterations (4,5) were found in the brown fat during the late stage following stress, which also supports the assumption that, in hamsters, cortisone is a relatively late-appearing product in the endocrine chain reaction initiated by stress. Possibly, a belated enhancement of viral multiplication subsequent to the belated hypercortisolemia initiated by stress is the mechanism by which disappearance of the virus is prevented in hamsters submitted to stress, in contrast with the controls.

Summary. 1) When MEF₁ poliomyelitis virus was introduced into the brown fat of hamsters treated with cortisone or submitted to stress there was viral persistence for periods of time significantly longer than in control animals. 2) During the initial 3 days following inoculation, viral multiplication rate was significantly enhanced in cortisone-treated but unchanged in stressed as compared to control hamsters. 3) The mechanism of the above difference as well as its effect upon the

development of experimental poliomyelitis is discussed.

The capable technical assistance of Mildred Steinglass and Guido Rio is thankfully acknowledged.

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Received May 14, 1957. P.S.E.B.M., 1957, v95.

Effects of Protein Depletion and Inanition on Serum Glycoprotein Concentrations in the Rat.* (23326)

HENRY E. WEIMER AND HISAKO NISHIHARA (Introduced by Charles M. Carpenter)
Department of Infectious Diseases, School of Medicine, University of California Medical Center, Los Angeles

Increases in the carbohydrate-containing proteins of serum have been reported in several pathologic states characterized by cachexia, such as cancer and tuberculosis(1). The suggestion that degradation of body tissues may be responsible for elevated serum glycoprotein levels(2) makes desirable experimental studies in which marked weight loss occurs but in which disease processes are not involved. If the catabolism of somatic tissues is responsible for increased serum glycoprotein levels, then nutritional states characterized by pronounced weight loss might be expected to cause an increase in the protein-bound carbohydrates of serum.

The present study is concerned with the effects of protein depletion and inanition on serum concentrations of total glycoprotein, serumucoid, albumin polysaccharide, globulin polysaccharide, total protein, albumin and globulin, and on the hemoglobin and hematocrit values of blood in adult, male, Sprague-Dawley rats.

Methods. The rats were housed singly in suspended type wire cages. They were maintained on a diet of Purina laboratory chow and tap water, *ad lib.* until initiation of the study. Blood samples were obtained by cardiac puncture under light ether anesthesia.

Normal Group. The group was composed of animals maintained under the conditions described above. *Protein Depletion Group.* Rats were fed a protein-free diet[†] consisting of 70% corn starch, 15% alphacel (cellulose), 10% vegetable oil, 4% salt mixture (U.S.P. XIV), 1% cod liver oil, and supplemented with the following vitamin mixture[†] (mg/kg): alpha tocopherol 110, ascorbic acid 1000, inositol 110, choline chloride 1660, menadione 55, p-aminobenzoic acid 110, niacin 100, riboflavin 22, pyridoxine hydrochloride 22, thiamine hydrochloride 22, Ca pantothenate 67, biotin 0.44, folic acid 2, Vit. B₁₂ 0.03, Vit. A 20,000 units/kg, and Vit. D 2,220 units/kg. The animals were fed the diet 20 days at which time they had lost approximately 25% of their original weight. *Inanition Group.* Food was withheld from the rats until they had lost 25% of their original weight, the depletion requiring 8 days. Drinking water was available at all times. *Serum and blood determinations.* Chemical and hematologic analyses were carried out by methods previously reported(3,4,5). The globulin fraction was isolated by an adaptation of the method of Pillemer and Hutchinson(5). The components of the albumin fraction were estimated by difference. An

* Supported in part by a research grant from National Cancer Inst.

[†] Obtained from Nutritional Biochemicals Corp., Cleveland.

TABLE I. Effects of Protein Depletion and Inanition on Serum Levels of Protein-Bound Carbohydrates and Proteins.

Group	A. Normal	B. Protein-free diet	C. Inanition
No. rats	20	20	20
Initial wt (g)		403 $\pm$ 5.7	407 $\pm$ 8.5
Final wt "	411 $\pm$ 5.2	305 $\pm$ 6.2 †	305 $\pm$ 6.2 †
Hematocrit (%)	45 $\pm$.4	47 $\pm$.4 †	(†) 53 $\pm$.8 †
Hemoglobin (g %)	14.7 $\pm$.17	14.7 $\pm$.11	(†) 16.5 $\pm$.28 †
Total serum glycoprotein (mg %)	166 $\pm$ 2.1	128 $\pm$ 2.2 †	132 $\pm$ 1.7 †
Albumin polysaccharide	19 $\pm$.9	19 $\pm$ 1.4	16 $\pm$ 1.4
Globulin polysaccharide "	147 $\pm$ 2.0	109 $\pm$ 2.4 †	(*) 116 $\pm$ 2.3 †
Seromucoid fraction "	27 $\pm$.6	23 $\pm$.6 †	(*) 21 $\pm$.6 †
A/G ratio (protein-bound carbohydrate)	.13 $\pm$.007	.17 $\pm$.016*	.14 $\pm$.015
Total serum protein (g %)	5.7 $\pm$.06	5.0 $\pm$.08 †	5.1 $\pm$.02 †
Albumin "	2.6 $\pm$.05	2.6 $\pm$.06	2.6 $\pm$.02
Globulin "	3.1 $\pm$.07	2.4 $\pm$.07 †	2.5 $\pm$.02 †
A/G ratio (protein)	0.8 $\pm$.04	1.1 $\pm$.04 †	1.0 $\pm$.05 †

Findings include stand. error of mean.

Statistically significant differences from normal values are indicated:

* $P = <.05 >.01$.

† $P = <.01$.

(*) and (†) indicate statistically significant differences between Groups B and C.

analysis of the data was made by standard statistical procedures, employing the *t* test (6).

Results. A summary of the data is presented in Table I. The results of the hematocrit determinations indicate that different degrees of hemoconcentration occurred in the experimental groups as a result of the dietary regimens. The effects were most pronounced in the fasted group.

Changes in the serum glycoprotein levels were comparable in both experimental groups. With the notable exception of the carbohydrate moiety of the albumin fraction, all other protein-bound carbohydrate values were significantly decreased.

The pattern of decrease of the serum proteins was identical to that of the protein-bound carbohydrates. The relative stability of the albumin fraction in acute depletion was demonstrated by the increased A/G ratios and may be a reflection of the slower rate of turnover of serum albumin(7). A similar observation has been reported for man(8).

The results of the present investigation are not in agreement with the hypothesis(2) that tissue degradation is primarily responsible for increased serum glycoprotein concentrations. Apparently other factors are involved. Winzler(1) has suggested the possibility of increased local synthesis in pathologic condi-

tions and has reviewed other hypotheses.

Summary. The effects of protein depletion and inanition on serum concentrations of total glycoprotein, seromucoid, albumin polysaccharide, globulin polysaccharide, total protein, albumin, and globulin and on hemoglobin and hematocrit values of blood have been investigated in adult, male Sprague-Dawley rats. Significant decreases were observed in all serum constituents with the exception of the albumin fraction. The response of the protein-bound carbohydrates of serum was similar to that of the serum proteins. A marked hemoconcentration occurred in the fasted group. The results are not in agreement with the hypothesis that tissue degradation is responsible for increased serum glycoprotein concentrations.

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Clin. Invest., 1949, v28, 273.

Received May 20, 1957. P.S.E.B.M., 1957, v95.

Diurnal Variations in Excretion of Chloride in Normal Subjects.* (23327)

STANLEY NOBLE (Introduced by W. B. Youmans)

Department of Physiology, University of Wisconsin, Madison

A number of investigators have reported a diurnal rhythm in the excretion of sodium or chloride. Most of these authors were interested in the effects of sleep(1-7). Some studied the salt excretion for consecutive time intervals of different lengths(8,9). The effects of posture(10) and of varying degrees of muscular work(11) also have been considered. In all studies most of the normal subjects excreted more sodium chloride/hour during the day than during the night. However, the number of cases which have been studied is relatively small. Since derangement of the diurnal rhythm in excretion of sodium chloride is sufficiently characteristic in certain diseases to be an aid in diagnosis, and possibly in prognosis and evaluation of therapy, more information is needed on the variations for normal subjects(10-13).

Methods. Forty-one normal subjects were studied, 40 of whom were between 18 and 33 years of age. The subjects were university students or laboratory workers. There was no regulation of their salt intake or their degree of activity. No attempt was made to standardize or in any way to regulate their sleeping and eating habits. They were required simply to furnish a consecutive day and night urine. Each subject recorded his time of retiring and of arising. The night urine was that produced during the recumbent period after retiring at night, and the day urine was that of the up-and-about period between arising and retiring. Chloride was determined by the Volhard-Harvey method. Chloride excretion is expressed as NaCl excretion/hour. NaCl excretion/hour

during the day divided by NaCl excretion/hour during the night is the ratio which is calculated.

Results. The chloride excretion/hour during the day, expressed as NaCl, ranged from .15 to .96 g with a mean of .48 g and standard deviation of .21. The chloride excretion/hour during the night, expressed as NaCl, ranged from .04 to .64 g with a mean of .25 g and standard deviation of .14. For the day to night ratios the range was 0.71 to 4.58 with a mean of 2.31 and standard deviation of 1.09. Creatinine excretion determined in 25 of the subjects ranged from 1.16 to 2.73 g per 24 hours(14).

The correlation coefficient for the total amount of salt excreted per 24 hours and the day-to-night NaCl excretion ratio was +0.026. The distribution of the ratios of the 41 subjects studied did not differ significantly from that of the ratios of 28 subjects for whom data necessary for calculation of the ratios had been reported by other investigators. The distribution of the day-to-night Na or Cl excretion ratios for the 69 subjects is shown in Fig. 1. The lowest ratio found

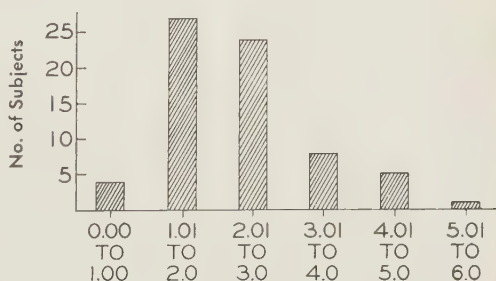


FIG. 1. Distribution of day-to-night NaCl excretion ratios of normal subjects.

* Aided by grant from Wisconsin Heart Assn. and funds for research assistantship from the Wisconsin Alumni Research Fnd.

in a normal subject was 0.71. This is to be contrasted with the low ratios seen in subjects with congestive heart failure, cirrhosis of the liver and orthostatic hypotension(10-12).

Summary. The day-to-night ratio of urinary chloride excretion has been determined for each of 41 normal subjects and has been calculated for 28 subjects reported in other papers. No restrictions of diet or activity were imposed. In the great majority of the subjects more chloride was excreted/hour during the up-and-about period than during the period of recumbency. This was true regardless of the amount of chloride excreted/24 hours.

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Received May 20, 1957. P.S.E.B.M., 1957, v95.

Mechanism of Reflex Bradycardia which Follows Sudden Closure of an Arteriovenous Fistula.* (23328)

DONAL M. BILLIG† AND OSCAR W. SHADLE (Introduced by H. C. Lawson)

Department of Physiology, University of Louisville School of Medicine, Louisville, Ky.

The bradycardia which follows sudden closure of an arteriovenous fistula was first described by Nicoladoni(1), and independently by Branham(2). There have since been numerous attempts to explain the mechanism of this response. Carotid-aortic pressoreceptors(3), pressoreceptors of the right atrio-caval junction(4), and reflexes arising in the vessels at the site of the fistula(5) have all been discussed as possible sites of origin of the reflex bradycardia. Lewis and Drury (6) showed that atropine will abolish the bradycardia, and concluded that the efferent limb of the reflex is the vagus. This work was later confirmed by Kramer and Kahn(7). The purpose of this report is to describe the afferent limb of the reflex and to define the mechanism which initiates the reflex bradycardia.

Methods. Dogs, weighing 11 to 27 kg,

*Supported in part by grant from Louisville and Kentucky Heart Assn.

† Louisville Heart Assn. Student Research Scholar.

were premedicated with 1-2 mg/kg of morphine sulfate, and anesthetized with 70-90 mg/kg of chloralose, intravenously. Heparin, 5 mg/kg, and Manuronate, 20 mg/kg, were used as anticoagulants. Heart rates were measured from electrocardiograms recorded on a Sanborn visocardiette, or in some dogs from systemic pressure pulse tracings recorded by a strain gauge or Gregg manometer. Systemic arterial pressure was measured through a brachial artery cannula, and in some experiments right atrial pressure was measured through a catheter passed into the atrium by way of the jugular vein. The carotid sinuses were denervated either by adventitial stripping and phenol painting, or by complete extirpation. The aortic arch was denervated according to a modification of the method of Comroe(8). The left vagus nerve was cut high in the neck, and all branches running medially and laterally from the right main vagal trunk were cut down to the level of the aortic arch, leaving the right main

TABLE I. Effect of Sudden Closure of an Arteriovenous Fistula on Heart Rate of 11 Dogs.

Before denervation		After denervation		Remarks
Control	Close	Control	Close	
194	164	210	214	Closed chest animal. Carotid sinus denervation only.
168	144	173	174	<i>Idem</i>
108	87	271	267	"
167	138	246	200	"
57	52	189	149	"
135	113	214	216	Open chest preparation. Carotid sinus and aortic arch denervation.
95	90	103	105	<i>Idem</i>
125	117	199	199	"
146	141	170	169	"
106	89	148	148	" . Aortic denervation only. Carotid sinus intact.
134	134	125	125	" . No carotid or aortic reflexes obtainable at any time.

vagal trunk with the right cardiac vagus intact. The functional integrity of the right cardiac vagus was established after the dissection by observing the effect on heart rate of electrical stimulation of the right vagus nerve high in the neck. Positive pressure respiration was used on all dogs. The arteriovenous fistula was made from brass, rubber and polyethylene tubing, arranged so as to connect both femoral arteries with a single femoral vein. The tubing connecting the arteries to the vein was manually closed with a hemostat. The carotid sinuses were stimulated by manual compression. Aortic arch compression could be done only in open chest dogs, where partial mobilization of the aortic arch had been accomplished by dissection of the right vagus. Pressure on the arch was made by means of long rubber-coated hemostats inserted through the thoracotomy incision.

Results. Fig. 1 is a segment of a record

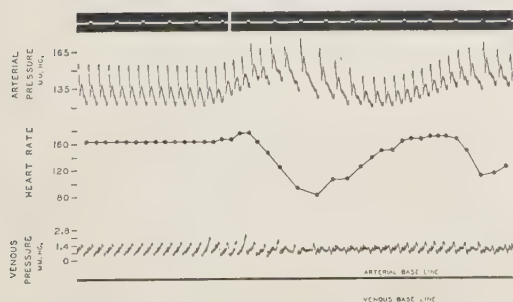


FIG. 1. Response in arterial pressure, heart rate (calculated), and venous pressure in right atrium to sudden closure of an arteriovenous fistula. Time in seconds, and instant of closure, above.

showing the typical response of the cardiovascular system to sudden closure of an arteriovenous fistula (Nicoladoni-Branham sign). The initial response is an instantaneous elevation of systolic and diastolic arterial pressures, accompanied by a bradycardia starting 2 to 4 beats later. These changes occur in the absence of any change in mean central venous pressure. Following marked initial slowing, the heart rate then tends to return toward its control value, and may fluctuate about a mean as a damped sine wave, which reciprocally follows similar waves in arterial pressure. Because of this secondary rise in heart rate, average values for heart rate over an interval of time may not give a true picture of the immediate effect of the sudden closure of a fistula.

The results of all the experiments are presented in Table I. The heart rates in the Table are the average of 10 beats prior to closure (control), and 10 beats immediately following closure (close) of the fistula. The first 5 animals show the effect of carotid sinus denervation alone on the bradycardiac response. It can be seen that simple carotid sinus denervation abolished the bradycardia in 2 animals and had no effect in 2 other animals. In dog 3, the small residual change in heart rate (271-267) is probably not significant at this high rate. Because of this residual bradycardia in 2 animals with carotid sinus denervation, in the next 4 animals both the carotid sinuses and aortic arch were denervated. As indicated in the Table, this procedure completely abolished the brady-

cardia. In dog 10, aortic arch denervation alone was successful in abolishing the afferent limb of the reflex, suggesting that either of these pressor areas may predominate in an individual animal. In dog 11 no reflex bradycardia, either from closure of the fistula or from compression of the carotid sinuses or aortic arch, could ever be obtained.

Discussion. Lewis and Drury(6) were the first to suggest that sudden closure of an arteriovenous fistula increased peripheral resistance, and that the bradycardia was a response to the increased arterial pressure, which invoked Marey's Law. Nickerson, Elkin and Warren(3) studied human subjects with arteriovenous fistulae, observed the same results as Lewis and Drury, and concluded that the bradycardia was initiated via the carotid-aortic pressoreceptors. Our data give experimental confirmation to the supposition that the afferent limb of this reflex bradycardia arises from the arterial pressoreceptors. After complete denervation, closure of a fistula causes the usual changes in arterial pressure, without the bradycardia.

In some animals the bradycardia was abolished by carotid sinus denervation alone, the aortic arch receptors remaining intact, while in another dog just the reverse was found. This indicates that in an individual dog either of the pressoreceptor areas may predominate, or that they may both be receptive. This variability in the relative predominance of two homologous receptor areas has been shown to exist for the aortic and carotid chemoreceptors(8).

The "rebound effect" in arterial pressure

and heart rate which follows quick closure of a fistula (Fig. 1) has been shown previously by Van Loo and Heringman(4). The same type of response to rapid arterial pressure changes has been demonstrated by Guyton and Harris(9), who abolished these waves by carotid-aortic pressoreceptor denervation. This "rebound effect" was abolished by denervation of the pressoreceptor areas in our work, offering further evidence that the afferent limb of the bradycardiac response is from the carotid sinuses and aortic arch receptors.

Conclusions. 1. The receptors for the reflex bradycardia which follows sudden closure of an arteriovenous fistula, are the pressoreceptors of the arterial side of the circulation, the carotid sinuses and aortic arch. 2. In some animals the carotid sinuses are the predominant arterial pressoreceptors, and in others the aortic arch is predominant.

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Received May 23, 1957. P.S.E.B.M., 1957, v95.

Propagation of Infectious Canine Hepatitis Virus in Cultures of Pig and Ferret Kidney. (23329)

A. HOWARD FIELDSTEEL* AND GEORGE M. YOSHIHARA

(Introduced by Isaac Ruchman)

Cutter Laboratories, Berkeley, Calif.

Propagation of infectious canine hepatitis virus (ICH) in cultures of canine renal epithelium has been reported(1,2). After 41 serial passages the virus became modified in its virulence for dogs(2), but retained its *in vivo* renal tropism. Even after 86 passages in kidney cultures plus 10 passages in canine uterus cultures all dogs that were tested excreted modified virus in the urine(3). Susceptible dogs placed in contact with vaccinated dogs developed an immunity to ICH without showing signs of disease. These also excreted modified virus. It was possible to produce inapparent infection by this method by 4 successive serial back-passages in dogs, but at the fifth passage the virus reverted to virulence and 2 of 3 exposed dogs developed a fatal ICH infection.

It seemed likely that if the carrier state were to be eliminated the virus would have to be propagated in tissues of other animals. Although ICH virus naturally infects members of the canine species only, it has been reported that corneal opacity typical of this disease could be produced in ferrets inoculated intraocularly with ICH virus(1). In our initial experiments equivocal evidence was obtained that unadapted ICH virus from canine tissues multiplied at a very low level in pig kidney cultures(2). Both of these observations seemed to warrant further investigation. We present evidence that ICH virus can be propagated in cultures of ferret and pig kidney with the production of consistent and typical cytological changes.

Materials and methods. Tissue cultures. The pig and dog kidney cultures were continuous lines of epithelial-like cells derived from cells cultivated originally by the method of Youngner(4). Stock cultures were carried in 2 and 5 l. Povitsky bottles. Cell suspensions

were made by replacing the medium with an equal quantity of buffered solution of 1:5000 versene (disodium salt of ethylene-diamine-tetracetate). After incubation at 36°C for 15-30 minutes, with occasional shaking, the cells peeled from the glass, and formed a homogeneous single cell suspension. The cells were centrifuged at 1,000 rpm for 5 minutes, the versene discarded and the cells resuspended in growth medium equivalent to twice the original volume. *Pig kidney* cells for virus propagation were distributed in 6 ml amounts to 2 oz. French squares or prescription bottles. Occasionally 8 oz. Blake bottles containing 50 ml of cell suspensions were used for cultivation. A complete monolayer of cells generally formed in 7-9 days. *Dog kidney* cells were used for virus titrations only and distributed in 2 ml amounts to 16 x 150 mm tubes. These cells had an extremely fast rate of growth, forming a complete sheet in 2-3 days; multiplication being observed after 8 hours of incubation at 36°C. *Ferret kidney* cultures were primary cultures of epithelial-like cells prepared from adult animals by the method of Youngner. Both roller tubes and bottle cultures were utilized. Otherwise maintenance of cultures and cultivation of virus was the same as for pig and dog kidney. *Media.* Growth media for all cells contained 0.5% lactalbumin hydrolysate and 0.2% yeast extract in Earle's balanced salt solution. However, the latter was modified by decreasing the NaHCO₃ content by 50% and adding 1.2 g/l of tris (hydroxymethyl) aminomethane(5). The pH was adjusted to 7.4 by addition of 1 N HCl. For cultivation of pig kidney cells the growth medium was supplemented with either pig or calf serum in final concentration of 20%. The dog kidney cells required only 5% calf, lamb or horse serum in addition to the growth media, while the ferret kidney grew best with a supplement of 20% lamb serum. After the cultures had

* Present address: Virus Laboratory, Montana State Board of Health, Helena.

formed complete monolayers, unless they were used immediately for virus propagation, serum concentration was maintained at 5%. For propagation of ICH virus all cultures were treated in the same manner. The growth medium was removed and replaced with equal quantity of phosphate buffered saline (PBS) which bathed the cells for 1 hour at 36°C. This was done to remove antiviral substances which might have been present in the serum. The PBS was then replaced by equal quantity of medium 199(6), pH 7.6. Serum was not used during virus growth phase and cultures could be maintained for periods to 2 weeks at 36°C. All media contained 200 units penicillin and 200 µg streptomycin/ml. *Virus titrations.* The amount of virus obtained from both pig and ferret cultures was determined by inoculation of decimal dilutions of infected fluids into roller tube cultures of dog kidney. Dilutions were prepared in medium 199 and 0.2 ml aliquots of each dilution were added to 4 tubes containing 1.8 ml of media. Cultures were rotated at 36°C and examined for characteristic cytologic changes daily for 10 days. Titters are expressed as number of tissue culture infectious doses/ml (TCID₅₀) as calculated by the method of Reed and Muench(7).

Results. Propagation of ICH virus in ferret kidney. Although virus obtained from different passage levels in dog kidney was propagated in ferret kidney, only the experiments carried out with virus from the 12th dog kidney passage will be reported. In the first passage in ferret kidney 1 ml of virus was inoculated into 8 oz. Blake bottles. Cytologic changes were observed after 48 hours, involving all cells by 72 hours. A second passage was made in similar manner from harvested fluids, but cytologic changes occurred slowly and involved only about 50% of the cells by the 7th day. Subsequent passages were made by inoculating undiluted culture fluid into roller tubes or French squares to give a final dilution of 1:10. Rarely was more than half the cell population affected. In 2 instances non-specific degeneration occurred after 14 days, before specific cytolysis was noted. It was necessary to go back to the previous passage and use the infected fluid

TABLE I. Results of Serial Passage of ICH Virus in Ferret Kidney Cultures.

Passage No.	Harvest (day after inoc.)	-Log of titer/ml
DK* 12		8.0
FK† 1	3	7.0
2	7	6.0
3	5	6.2
4	5	5.7
5	7	4.4
6‡	8	N.D.
7	10	N.D.
8	8	5.0
9	7	4.4
10	8	4.5
11‡	9	5.0
12	4	6.2+
13	4	N.D.
14	7	5.0
15	6	5.4
16	8	4.9
17	6	4.7
18	11	5.2

* Dog kidney. † Ferret kidney. N.D., not done.

‡ Harvested fluids from these passages were used as sole source of nutrient and virus inoculum for succeeding passage. In all other passages final dilution of inoculum was 1:10 in fresh nutrient fluid.

as the sole source of both virus and nutrient fluid. This was done with both the 7th and 12th passages and it will be noted from Table I that harvest of these fluids was carried out at 10 and 4 days respectively when at least 50% of the cells showed specific changes. Although virus yields were greater in dog kidney than in ferret kidney there is little doubt that the virus propagated in the latter. Virus present in the 18th ferret passage represented a cumulative dilution of the original inoculum of 10^{-22.6}.

Propagation of ICH virus in pig kidney cultures. Cytologic changes invariably occurred 2-3 days after inoculation of undiluted virus into bottle cultures where final dilution was 1:50 in the initial passage and 1:10 in each of the following passages. Although the changes observed were different from those observed in either the dog or ferret kidney they were readily recognizable and characteristic of ICH virus in this tissue. Fluids from each passage were harvested when 50 to 100% of the cells showed signs of infection which was generally 1 to 4 days after initial observation of cytologic changes. The results of 11 serial passages of ICH virus in pig kidney are summarized in Table II. While the

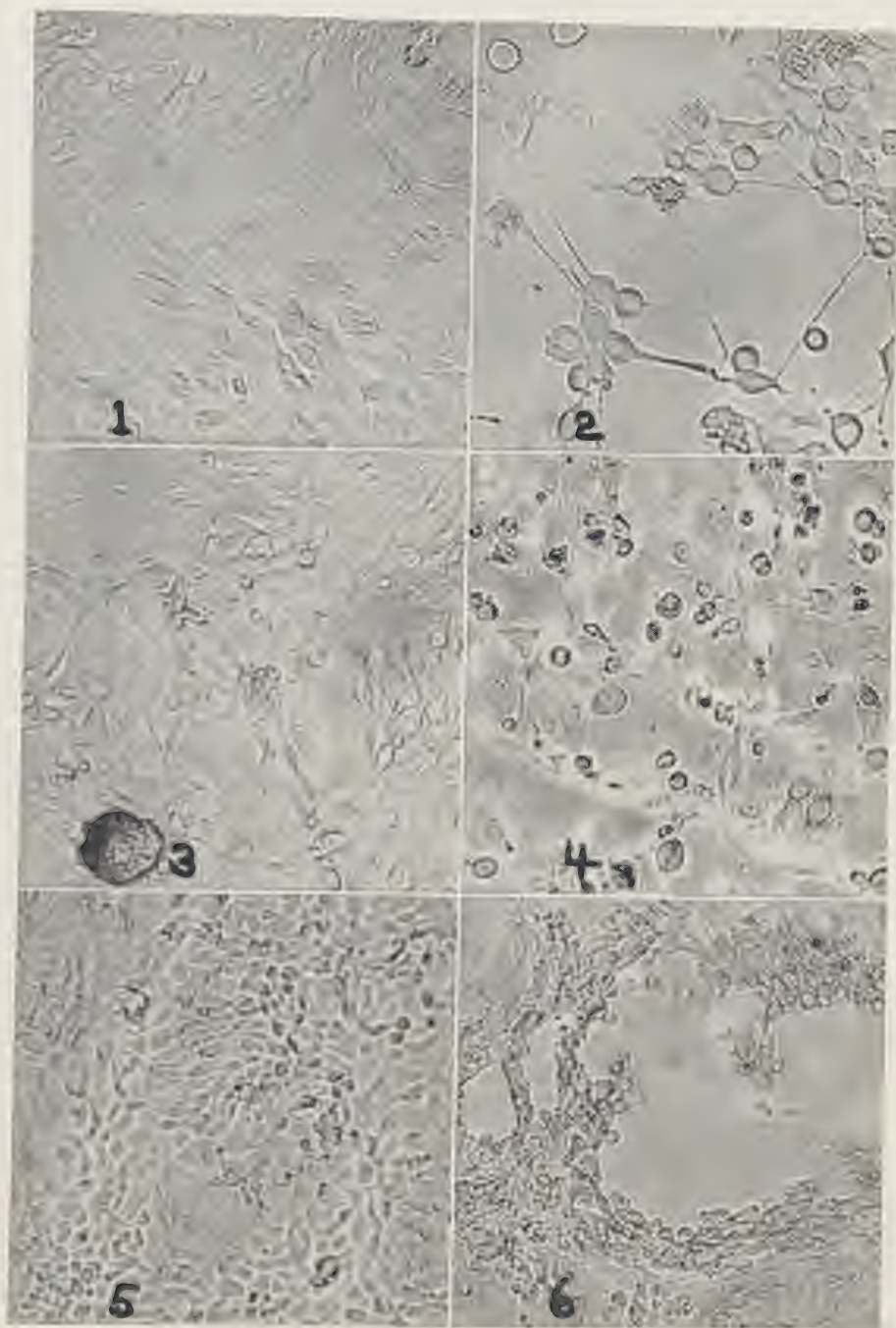


FIG. 1. Continuous culture of normal dog kidney.

FIG. 2. Cytologic changes in dog kidney culture 3 days after inoculation with 10^6 TCID₅₀ of ICH virus.

FIG. 3. Primary culture of normal ferret kidney.

FIG. 4. Cytologic changes in ferret kidney 6 days after inoculation with 10^5 TCID₅₀ of ICH virus.

FIG. 5. Continuous culture of normal pig kidney.

FIG. 6. Cytologic changes in pig kidney 4 days after inoculation with 10^5 TCID₅₀ of ICH virus. (All figures 100 $\times$.)

TABLE II. Results of Serial Passage of ICH Virus in Pig Kidney Cultures.

Passage No.	Harvest (day after inoc.)	-Log of titer/ml
DK* 12		8.0
PK† 1	5	N.D.
2	7	5.2
3	6	5.7
4	4	N.D.
5	4	5.4
6	4	6.2
7	6	7.0
8	4	7.2
9	4	6.7
10	3	6.0
11	7	7.2

* Dog kidney. † Pig kidney. N.D., not done.

titers are higher than those obtained from virus passaged in ferret kidney, they are not as high as those obtained from dog kidney passaged virus. However, they are consistent with virus multiplication since fluids harvested from the 11th passage represented theoretical virus activity at cumulative dilution of the original inoculum of $10^{-18.1}$.

The ready adaptability of ICH virus to continuous cultures of pig kidney was demonstrated in the line of virus which had been passaged both in dog and ferret kidney. Virus from the 13th ferret kidney passage was passed serially in pig kidney cultures and consistently produced cytologic changes in 2 to 3 days with high titers of virus by 3 to 7 days after inoculation (Table III). Virus activity from the 10th passage of this series represented a cumulative dilution from the original dog kidney virus of $10^{-29.1}$. For comparative purposes cytologic changes produced by ICH

TABLE III. Results of Passage of Ferret Kidney Adapted ICH Virus in Cultures of Pig Kidney.

Passage No.	Harvest (day after inoc.)	-Log of titer/ml
DK* 12		N.D.
FK† 13		N.D.
PK‡ 1	7	5.7
2	6	5.7
3	4	N.D.
4	4	6.2
5	4	6.2
6	6	7.0
7	4	6.7
8	4	6.5
9	3	7.2
10	7	7.2

* Dog kidney. † Ferret kidney. ‡ Pig kidney. N.D., not done.

virus in dog, ferret and pig kidney are illustrated in Fig. 1-6. Control cultures are of the same age as the inoculated ones and received the same treatment with the exception of virus inoculations. In no instance were similar cytologic changes noted in control cultures.

Identification of the 3 lines of passage viruses was established by neutralization tests carried out in dog kidney cultures. Pooled serum from dogs known to be susceptible to ICH virus failed to neutralize any of the passage viruses. However, serum from these same dogs obtained after they had undergone active infection produced by known ICH virus neutralized each of the 3 passage viruses. Furthermore, 4 dogs which were inoculated with ferret kidney passaged virus developed antibodies against known dog kidney passaged virus and resisted challenge with virulent ICH virus.

The work reported here has increased the host range of ICH virus to tissues of species totally unrelated to the dog. However, it remains to be determined if on continuous serial passage in either or both ferret and pig kidney the virus will lose its *in vivo* renal tropism in dogs so that it may be used safely in modified form as an immunizing agent.

Summary. Infectious canine hepatitis virus, a hitherto highly host specific virus has been propagated in primary cultures of ferret kidney and in continuous cultures of pig kidney, virus multiplication being accompanied by distinctive cytologic changes. The virus was identified by neutralization tests with specific antiserum.

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Received May 23, 1957. P.S.E.B.M., 1957, v95.

Means of Increasing the Tolerated Dose of Isoniazid in Mice. II. Certain Amino Acids. (23330)

BENJAMIN PRESCOTT, GLADYS KAUFFMANN, AND WALTER D. JAMES
(Introduced by R. J. Huebner)

U. S. Department of Health, Laboratory of Infectious Diseases, Nat. Inst. of Allergy and Infectious Diseases, N.I.H., Bethesda, Md.

The acute oral toxicity of isoniazid, an effective therapeutic agent in human tuberculosis, has been reported to be reduced in mice by simultaneous administration of appropriate amounts of glycine and sodium glucuronate(1), or of cycloserine(2), or glycerine(3). The present investigation extends the study to the effect of certain other amino acids on isoniazid (INH) oral toxicity in DBA mice.

Methods. The minimal lethal dose of INH by oral administration in DBA mice is 6 mg/20 g mouse (300 mg/kg). However, since glycine and sodium glucuronate permitted the survival of mice given repeated administrations of a 10 mg dose of INH, this amount of INH (500 mg/kg) was adopted for the initial tests with other amino acids. As with glycine, the amount of amino acid used for first trial was approximately 5 or 6 millimols/millimol of INH. The chemicals were weighed, mixed in one tube, and dissolved or suspended in water to such a volume that the desired concentration of each was contained in 0.5 ml for oral administration. When necessary, hydrogen ion concentration was adjusted to approximately pH 5. Due to the low solubility in water of the desired concentrations of tryptophane, leucine, isoleucine, these amino acids were unsuitable for oral administration and were omitted from the series. This study is limited to the use of water as solvent; results of investigation of other solvents will follow later. The series of experiments include (a) results of mouse survival tests with 10 mg INH and each of 26 amino acids; (b) results with 8 mg INH and 11 of these effective acids; (c) repeated oral administrations of various amounts of L-cysteine-HCl with INH; (d) blood plasma levels after a single administration of various amounts of L-cysteine-HCl and INH; and (e) *in vitro* tests on the effect of 4 amino acids on the bacteriostatic action

of INH on one strain of avirulent *Mycobacterium*. Toxicity tests were made in 5 DBA mice for each of the amino acids studied in the amount that would represent a 5:1 molar ratio with INH. With the single exception of DL-aspartic acid with which one mouse died, all the other amino acids permitted 100% survival. It is of interest that a 10:1 proportionate amount of L-cysteine-HCl permitted 90% survival of a single dose.

Results. *Mouse survival after a single oral administration of isoniazid with amino acid.* With 10 mg of INH only 4 of these 26 amino acids were consistently able to protect 50% or more of the mice (Table I). Although in one test several of the other acids gave this result—L-serine, DL-serine, L-histidine-HCl, DL-aspartic acid, L-arginine-HCl, DL-threonine, DL-valine, DL-methionine, L-ornithine-HCl—duplication was not obtained in a second or third test. Of the 4 effective amino acids, only L-cysteine-HCl gave consistently better than 50% survival in repeated tests. In most instances increasing the amount of any of these acids beyond the 5:1 molar ratio with this dose of INH did not increase the percentage of survivors.

As also shown in Table I, a dose of 8 mg INH was tested with 11 of the 26 amino acids in at least 5:1 molar amounts, or in the case of L-cysteine-HCl with a series of 6 concentrations of from 10 to 116 mg per 20 g mouse (approximately 1 to 12 mmols/mmol INH). As might be expected, there was a significant increase in survival rate. Three of the series which were only 20% or less protective with the 10 mg dose of INH were observed to effect 50% survival when tested with 8 mg INH; and 70% or more of the mice survived with hydroxy-L-proline, L-aspartic acid, L-histidine-HCl, L-glutamic acid-HCl, and with L-cysteine-HCl in 4 different concentrations, 30, 46, 58 and 87 mg per mouse. As a conse-

quence, L-cysteine-HCl was selected for further study.

Effect of repeated administration of various amounts of L-cysteine-HCl on INH toxicity in mice. Several experiments on repeated administrations of INH with L-cysteine-HCl have shown that, with daily administrations of 8 mg INH per mouse, there was an optimal concentration of 30 to 58 mg of L-cysteine-

HCl. Increasing or decreasing the amino acid concentration gave less satisfactory results. The best procedure, administration of 8 mg INH with 58 mg of L-cysteine-HCl at 72-hour intervals, permitted 60% survival after 15 doses. With 10 mg INH and 58 mg L-cysteine-HCl given at 72-hour intervals, 50% of the mice survived 7 doses. However, a similar mixture with 50 mg supplementary

TABLE I. Effect of a Single Oral Dose of Certain Amino Acids on Isoniazid Toxicity in DBA Mice.

Amino acid	Amino acid, mg*	Isoniazid—10 mg	Amino acid, mg*	Isoniazid—8 mg
		No. mice surviving No. mice tested		No. mice surviving No. mice tested
0		0/20		1/35
L-Cysteine-HCl	116	0/10	116	3/10
"	87	6/10	87	9/10
"	58*	56/75	58	20/20
"			46*	20/20
"			30	9/10
"			10	6/10
L-Glutamic acid-HCl	100	16/30	100	7/10
"	66*	11/20	53*	17/20
D-Glutamic acid	60	4/10		
DL-Glutamic acid-H ₂ O	60*	0/10		
L-Aspartic acid	48*	10/20	48	4/10
"			39*	7/10
DL-Aspartic acid	48*	5/30	48	10/20
"			39*	4/10
Hydroxy-L-proline	48*	10/20	39*	7/10
L-Arginine-HCl	76*	10/29	76	9/20
"			61*	6/10
L-Histidine-HCl	68*	8/30	68	14/20
"			55*	8/10
DL-Methionine	54*	6/26		
Acetyl-DL-methionine	96	15/30	70	11/20
"	70*	4/30	56*	26/40
L-Ornithine-HCl	61*	6/30		
"-diHCl			60*	13/20
DL-Ornithine-HCl	61*	2/10	49*	5/10
DL-Serine	38*	6/21		
L-Serine	38*	6/30	38	0/20
"			30*	10/20
DL-Threonine	43*	6/20		
DL-Valine	43*	6/20		
DL-Alanine	32*	2/10		
Beta-Alanine	32*	2/10		
DL-Citrulline	63*	2/10		
L-Proline	42*	2/10		
Glycine	27*	2/20		
L-Cystine	88*	1/10		
L-(+)-Lysine-HCl	67*	1/20		
DL-Homocystine	97*	0/6		
L-Tyrosine	66*	0/10		

* Indicates amt/20 g mouse to give a ratio of 5 mMols of amino acid/mMol isoniazid.

sodium glucuronate permitted 50% of the mice to survive 10 doses. This endpoint is in contrast to 70% survival after 15 doses of a mixture of 10 mg INH with 50 mg glycine and 50 mg sodium glucuronate(1). The difference may be partially accounted for by the excess of glycine (a 10:1 molar ratio), but the toxicity of L-cysteine-HCl *per se* would preclude the repeated use of such an amount of this acid. Thus, whereas a single administration of 8 mg INH alone permitted survival of only 2 of 70 animals, when supplemented with L-cysteine-HCl (58 mg), up to 15 doses given twice weekly were tolerated by 60% of the mice.

Blood plasma concentrations. Blood plasma concentrations of INH in mice were determined at different time intervals after combined INH-cysteine administration. The method of INH assay was the "pyridyl" procedure(4). In a control test, no interference in the INH color reaction was observed when L-cysteine-HCl was present. The highest level obtained was 7.8 mg% of INH in the plasma 1 hour after administration of a mixture of 8 mg INH and 58 mg L-cysteine-HCl. There was but little difference in the concentrations after either 8 or 10 mg INH with 58 mg L-cysteine-HCl either at the 2-hour interval—4.5 and 5.0 mg% respectively—or at the 24-hour interval—0.6 and 0.3 mg% respectively. However, the use of supplementary sodium glucuronate (50 mg) with 10 mg INH and 58 mg L-cysteine-HCl resulted in a lower 2-hour level and a somewhat higher 24-hour level—3.3 and 1.1 mg%. This latter observation may indicate an explanation for the longer survival time in this group than in the mice without glucuronate. As reported earlier, a control of 5 mg INH alone in DBA mice showed a 30-minute level of 6.5 mg%, a 2-hour level of 2.9 mg%, and little or no detectable amount after 24 hours. Hence it would appear that the INH-L-cysteine-HCl mixture produced somewhat higher levels which persisted longer than those produced by the highest tolerated dose of INH alone.

Effect of certain amino acids on INH bacteriostatic action. Following a procedure previously described(1), 4 of the series of amino acids, acetyl-DL-methionine, DL-ser-

ine, L-glutamic acid-HCl, and L-cysteine-HCl, were selected for an *in vitro* study of their possible effect on inhibition by INH of the growth of an avirulent *Mycobacterium* strain B103. These 4 acids in *in vivo* toxicity tests ranged in effectiveness from 13% to 100% (Table I). Yet none of them in 5:1 molar ratios altered the inhibition endpoint of INH, 5 to 10 μ g; nor were they *per se* inhibitory of growth of this microorganism. In addition, in 3 tests L-cysteine-HCl supplemented with sodium glucuronate in the proportions used in the repeated administrations experiment had no effect on the INH bacteriostatic action.

Discussion. A comparison of the results obtained here with L-cysteine-HCl and those with glycine shows that *per se* cysteine is more effective as a means of increasing the tolerated dose of INH. Thus with 10 mg INH, glycine permits only 10% survival, whereas L-cysteine-HCl permits 74% survival; and correspondingly with 8 mg INH, the survival rates are 50% and 100% respectively. Thus, in the 5:1 molar ratio in mice, L-cysteine-HCl alone permits tolerance of an INH dosage which would require supplementary sodium glucuronate if glycine were the amino acid of choice. These results would further indicate that many of the amino acids might prove effective in reducing the acute toxicity of the LD₅₀ dose of 5 mg INH, or even reducing the toxic manifestations due to repeated administrations of a sublethal dose.

The mechanism by which the animal is able to tolerate toxic drugs when amino acids are given concomitantly has been discussed by various workers. Martin and his collaborators(5), employing amino acids to reduce the toxic effect in mice of sulfa drugs and various anthelmintics, considered the effect a prophylactic one. Ragno(6), who found L-glutamic acid efficacious in INH therapy of certain human tuberculous infections, suggested that its effectiveness might be due to its combination with the ammonia released from INH *in vivo*. Support for this suggestion is found in the recent reports of Greenstein and collaborators(7) on metabolism of amino acids in rats, with the statement that

L-arginine is an efficient means of detoxifying ammonium acetate; whereas the present study suggests that arginine is not adequately effective with INH. Possibly amino acids may counteract the inhibitory effect of INH on such enzymes as diphosphopyridinenuclease, or on the diphosphopyridine nucleotide itself.

One clinical report has indicated that use of simultaneous glycine and sodium glucuronate with INH did not control peripheral neuritis, but in 2 tuberculous patients markedly reduced frequency of convulsive seizures induced by the prolonged INH therapy and thus permitted uninterrupted treatment(8). Since L-cysteine-HCl may be effective due to a different mechanism than glycine and sodium glucuronate, it is conceivable that its use might benefit those INH-treated patients in whom toxic reactions are not counteracted by glycine and sodium glucuronate.

Summary and conclusions. Twenty-six amino acids have been tested for their ability to reduce the toxic effect of isoniazid (INH) in mice. Eleven of these—L-cysteine-HCl, L-glutamic acid-HCl, L-histidine-HCl, L-aspartic acid, hydroxy-L-proline, L-ornithine-HCl, acetyl-DL-methionine, L-arginine-HCl, DL-aspartic acid, L-serine, and DL-ornithine-HCl—in appropriate concentration (a 5:1 molar ratio) permitted at least 50% survival of DBA mice given an 8 mg lethal dose of INH per 20-g mouse (400 mg/kg). Optimal

results were obtained with single or repeated administration of 58 mg of L-cysteine-HCl (2900 mg/kg) with 8 mg INH. This dosage yielded a maximum blood plasma level of 7.8 mg% 1 hour after administration, as contrasted with 6.5 mg% following a 5 mg dose of INH alone. No interference by 4 amino acids, L-cysteine-HCl, L-glutamic acid-HCl, DL-serine, or acetyl-DL-methionine, was noted in the INH bacteriostatic test on one strain of avirulent *Mycobacterium* B103. From this study it would appear that L-cysteine-HCl, and perhaps other amino acids, are as effective as glycine and sodium glucuronate, previously reported, in increasing the tolerated dose of INH in DBA mice.

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Received May 27, 1957. P.S.E.B.M., 1957, v95.

Bile Constituents of the Opossum *Didelphys marsupialis virginiana*.* (23331)

FUMIO NAKAYAMA, AND CHARLES G. JOHNSTON

Department of Surgical Research, Wayne State University College of Medicine, and
Detroit Receiving Hospital

In the study of gallstone disease the usual laboratory animal is not satisfactory because of low concentration of cholesterol in bile of the animals (Table I). It would be advantageous to find an animal which excretes a

large amount of cholesterol in the bile and in high concentration. Wibler(6) reported significantly high cholesterol content in opossum gallbladder bile. This report is based on the several constituents of the gallbladder and hepatic bile of the opossum. Bile acids in opossum gallbladder bile were first studied by Haslewood and Wootton(7), and cholic acid

* Aided by grants from Parke, Davis & Co., the N.I.H., U. S. Public Health Service, and Research Corporation of Receiving Hospital.

TABLE I. Cholesterol Content of Gallbladder Bile* of Different Species.

Species	Cholesterol conc.	Literature
Human	623	Isaksson(1)
Ox	29 (57)†	" (2)
Dog	120 (79)†	" (2)
Hog	100-350 (165)†	Doyon and Dufourt(3)
Rabbit	100-120 (70)†	Parhon and Werner(4)
Rat‡	13.5	Byers and Friedman(5)

* Variable on account of concentrating function of gallbladder.

† () are authors'.

‡ Hepatic.

was found as its major bile acid. In the present study, chenodeoxycholic acid was identified as another major bile acid beside cholic acid by paper chromatography according to Sjövall(3). It was found that the bile acids in opossum bile are conjugated with taurine. No glycine conjugated bile acid was found.

Methods. The opossum† was anaesthetized with ether and laparotomy was performed. Gallbladder bile was aspirated by syringe before cholecystectomy was done. The small size polyethylene tube was inserted in the common duct and the bile drained continuously for 24 to 48 hours. Cholesterol in bile was determined according to the method of Foldes (9), and Foldes and Wilson(10). Cholic acid was determined by the method of Irvin, Johnston and Kopola(11). Chenodeoxycholic acid was determined according to the method of Isaksson(12). Deoxycholic acid content was determined according to Szalkowski and Mader(13) from Isaksson's hydrolyzed ex-

tracts of the bile mentioned above. The content of phospholipids was obtained by multiplying the value of organic phosphorus by 25. Organic phosphorus was determined by the method of King(14). For extraction preparatory to the phosphorous determination, a mixture of 3 parts ether and one part ethanol was used. Bile salts used for paper chromatography were extracted and purified according to the method of Haslewood and Wootton (7). Unhydrolyzed and hydrolyzed bile acid extracts were subjected to paper chromatography according to Sjövall(8). Bile acids on the paper chromatogram were located with the use of antimony trichloride reagent under an ultraviolet lamp according to the method of Carey and Bloch(15). Identical samples of several bile acids used in paper chromatography were prepared as follows. Cholic acid (Matheson-Coleman) was used after one recrystallization from ethanol (m.p. 198°). Because of the difficulty of obtaining the pure deoxycholic acid, chenodeoxycholic acid, glycocholic acid and taurocholic acid, they were synthesized. Deoxycholic acid was synthesized from cholic acid by the method of Fieser and Rajagopalan(16) (m.p. 172°). Chenodeoxycholic acid was synthesized from cholic acid according to the method of Fieser and Rajagopalan(17) (m.p. 142°). Glycocholic acid and taurocholic acid were synthesized by the method of Cortese and Bauman (18).

TABLE II. Cholesterol, Bile Acids and Phospholipids Content of Opossum Bile.

	Cholesterol, mg %	Bile acids, mg/ml			Phospholip- ids, mg/ml
		Cholic	Chenodeoxy- cholic	Deoxy- cholic	
<i>Hepatic bile</i>					
1	41.1	1.9	2.0	.48	13.6
2	19.2	1.6	2.9	.55	9.6
3	8.9	1.3	1.9	.46	4.2
Mean	23.1	1.6	2.4	.50	9.1
<i>Gallbladder bile</i>					
1	259				
2	50.8	28.0			33.8
3	104	22.9	13.2	5.1	21.1
Mean	137.9	25.5	13.2	5.1	27.5
Wibler(6)	635				1.42

† These were supplied by Michigan Department of Conservation.

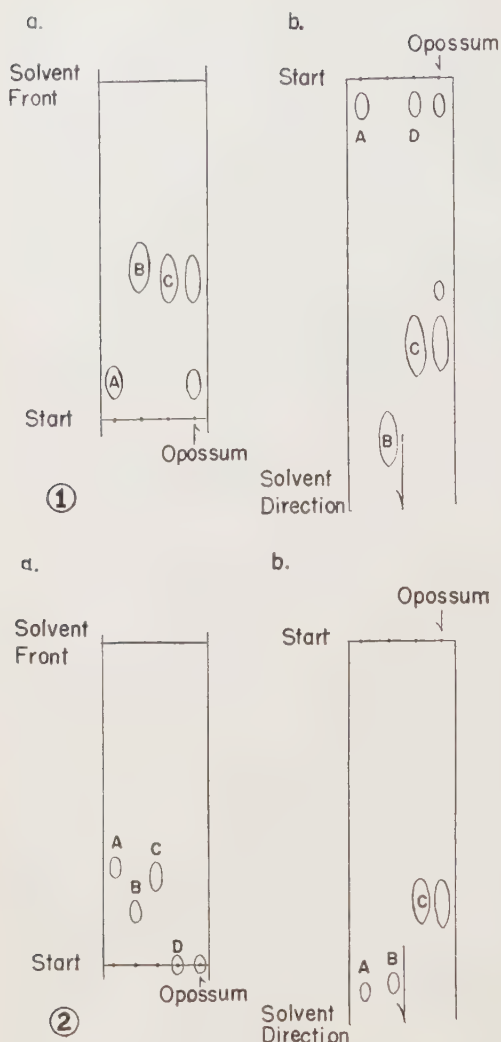


FIG. 1. Paper chromatograms of hydrolyzed bile acids of opossum. a. Ascending, 4 hr, moving phase: Isopropyl ether: heptane 60:40 v/v. Stationary phase: 70% acetic acid. Acids: Cholic (A), deoxycholic (B), chenodeoxycholic (C). b. Descending, 18 hr. Moving phase: Isopropyl ether: heptane 20:80 v/v. Stationary phase: As 1a. Acids: Cholic (A), deoxycholic (B), chenodeoxycholic (C), impurity in chenodeoxycholic (D).

FIG. 2. Paper chromatograms of unhydrolyzed bile acids of opossum. a. Ascending, 4 hr. Moving phase: Isopropyl ether: heptane 80:20 v/v. Stationary phase: 70% acetic acid. Acids: Cholic (A), glycocholic (B), glycocodeoxycholic (C), taurocholic (D). b. Descending, 12 hr. Moving phase: n-butanol saturated with 3% acetic acid-water v/v. Stationary phase: As 2a. Acids: Cholic (A), glycocholic (B), taurocholic (C).

Results of determinations of several constituents of opossum bile are summarized in

Table II. Cholesterol content of both gallbladder and hepatic bile is not as high as reported by Wibler(4), but is as low as that of usual laboratory animals. The relatively high concentration of chenodeoxycholic acid is remarkable. Isaksson's hydrolyzed extract(10) was also subjected to the determination of deoxycholic acid according to the method of Szalkowski and Mader(11). It has to be mentioned that the result of the amount of deoxycholic acid determined by this method is influenced by the existence of the large amount of other bile acids, *i.e.*, cholic acid, et cetera(11).

The method of paper chromatography according to Sjövall has been used to trace the bile acids. Fig. 1 shows the spots corresponding to cholic acid (Fig. 1a) and chenodeoxycholic acid followed by one unidentified spot (Fig. 1b). No spot corresponding to deoxycholic acid was found. In the system used in Fig. 2a, free and glycine conjugated acids run faster than taurine conjugated acids which are practically on the starting line, whereas bile acids of the opossum remain on the starting line. No spot corresponding to free and glycine conjugated acid was found. Fig. 2b shows the spot corresponding to taurine conjugated acid, *i.e.*, taurocholic acid. Free and glycine conjugated acids all run faster and practically with the front.

As mentioned above, chenodeoxycholic acid was identified as another major bile acid besides cholic acid which was isolated by Haslewood and Wootton from opossum bile(5). It was found that opossum bile contains taurine conjugated bile acids.

Summary. Several components of opossum bile were analyzed. Cholesterol content is comparable to that of other laboratory animals. Chenodeoxycholic acid was identified as another major bile acid besides cholic acid. Opossum bile contains taurine conjugated bile acids. No free and glycine conjugates were found.

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Received May 28, 1957. P.S.E.B.M., 1957, v95.

Carbohydrate Metabolism in Experimental Nephrosis (23332)

N. KALANT, M. CLAMEN AND M. M. HOFFMAN

(Introduced by J. S. L. Browne)

Departments of Medicine and Investigative Medicine, McGill University, and Research Laboratory, Jewish General Hospital, Montreal

It has been suggested that the nephrotic syndrome may be considered as "protein diabetes," the primary defect being loss of protein in the urine, and other biochemical abnormalities occurring as a consequence of this (1). In support of this view, Drabkin and Marsh(2) reported decreased liver glycogen levels and marked hypoglycemia on fasting in experimental nephrosis, changes interpreted as evidence of decreased gluconeogenesis consequent on the proteinuria. However, these observations were made under conditions which make interpretation difficult: (a) food intake was not controlled, (b) the animals were acutely ill and would have died within a few days(3). To investigate these results, a study has been made of carbohydrate metabolism in experimentally nephrotic animals after the acute stage, while on a fixed food intake.

Materials and methods. Adult male Sprague-Dawley rats weighing 380-420 g were used. They were fed twice daily by gavage with a diet providing a daily allowance of 55 calories, and containing 57% carbohydrate, 24% protein and 15% fat. There was free access to water. Animals were housed in individual metabolism cages in a room maintained at $26 \pm 1^\circ\text{C}$ and $40 \pm 2\%$ relative

humidity. Urine was collected under toluene. Post-absorptive blood glucose, 24-hour urine glucose, glucose tolerance and insulin sensitivity were measured before and at intervals after induction of nephrosis. Each animal thus served as its own control. Blood sugar measurements were made on 5 animals subjected to a 48-hour fast after 28 days of nephrosis. Since the animals had not been subjected to such a procedure during the control period, a new group of 6 normal rats of the same weight range were adapted to tube feeding and then fasted for 48 hours. Nephrosis was produced with anti-kidney serum by the method of Heymann and Lund(4). The daily protein excretion was 400-700 mg. Intravenous glucose tolerance tests were performed by injecting 1.5 g of glucose/kg body weight in 50% solution; tail tip blood was sampled 5, 10, 15, 20 and 30 minutes later. A plot of the log of blood sugar concentration against time gave a straight line from which the half-life of the injected glucose was obtained. To test insulin sensitivity 0.1 unit of commercial insulin/kg body weight was injected intravenously; blood sugar was measured at 0, 15, 30, 45, 60, 90 and 150 minutes after injection and the values plotted against time. Two

TABLE I. Urinary Excretion of Nitrogen and Glucose in Nephrosis. N intake—360 mg/24 hr.

	Control	0-5	6-10	Days after NTS			
				11-15	16-20	21-25	26-30
Nitrogen (mg/day)	341*	393*	385*	321	345	322	331
Glucose	13*	15*	14*	11	10	9	9

* Mean values derived from 8 animals. Others are mean values derived from 5 animals.

criteria of insulin effect were used; (a) fall in blood sugar concentration at 15 minutes (maximum fall almost invariably occurred at this time), and (b) area contained between the curve and a baseline drawn at the level of initial concentration. Urinary nitrogen was measured daily in 3 animals for 10 days and in 5 animals for 30 days after the production of nephrosis. Chemical measurements were made according to the following methods: (a) glucose—Somogyi modification(5) of the Nelson Method(6); (b) blood urea nitrogen—Schales(7); (c) nitrogen—semi-micro Kjeldahl procedure; (d) proteins—biuret procedure using the reagent of Gornall *et al.*(8); (e) serum cholesterol—Zlatkis *et al.*(9).

Results. The results are shown in Tables I-IV. Some animals died or were sacrificed during the experiment; data obtained within 2 days of death have not been included. Table II contains data which confirm the presence of the characteristic hypoproteinemia and hypercholesterolemia with transient elevation of the blood urea concentration.

Urinary glucose excretion was not affected by the presence of nephrosis. Post-absorptive blood sugar concentrations showed a small but statistically significant fall ($p < .01$). The half-life of intravenously administered glucose rose during the early stage of nephro-

sis, but then fell to or below control levels. These changes were significant at the 5% level. Similarly the fall in blood sugar produced by insulin was increased in the early period of nephrosis ($p < .05$) and later returned to normal. The areas under the blood sugar-time curve after insulin showed the same trends, but the changes were not significant at the 5% level.

Nitrogen excretion was increased during the first 10 days of nephrosis but then returned to control levels.

The response of nephrotic animals to a 48-hour fast was essentially the same as that of control animals.

Discussion. Evidence indicating abnormal carbohydrate metabolism in nephrosis is of 2 types: (a) Drabkin and Marsh(2) concluded on the basis of low liver glycogen levels and fasting hypoglycemia, that there was a decrease in gluconeogenesis in experimental nephrosis of rats, and (b) Gottfried, Steinman and Kramer(10) found flat oral glucose tolerance curves in nephrotic children. The animal studies, however, were obtained during early, fulminating nephrosis not comparable to the chronic disease of humans. The results of Gottfried *et al.* in children might be taken to indicate an increased rate of glucose utilization, but alterations in rate of glucose ab-

TABLE II. Changes in Blood and Serum Constituents in Nephrosis.

Constituent	Control	0-10	Days after NTS inj.		
			11-20	21-30	31-40
Blood sugar (mg/100 ml)	116 $\pm$ 1.7 (16)	103 $\pm$ 3.7 (16)	107 $\pm$ 5.2 (10)	102 $\pm$ 4.1 (5)	100 $\pm$ 5.8 (4)
Serum proteins (g/100 ml)	7.2 $\pm$.19 (10)	5.2 $\pm$.19 (10)	5.5 $\pm$.22 (7)	5.6 $\pm$.22 (4)	5.5 (2)
Serum cholesterol (mg/100 ml)	103 $\pm$ 4.6 (10)	435 $\pm$ 55 (9)	320 $\pm$ 68 (7)	547 $\pm$ 188 (4)	403 (2)
Blood urea nitrogen (mg/100 ml)	14.3 $\pm$.5 (14)	34.6 $\pm$ 5.3 (13)	18.3 $\pm$ 2.5 (8)	14.2 $\pm$ 1.6 (4)	

Values are for mean and stand. error of mean.

Figures in parentheses indicate No. of animals from which values obtained.

TABLE III. Effect of Nephrosis on Tests of Carbohydrate Metabolism.

Criterion	Control	Days after NTS	
		6-12	26
Fall in blood sugar 15 min. after insulin (mg/100 ml)	38 $\pm$ 5.2 (11)	49 $\pm$ 6 (11)	38.4 $\pm$ 7.9 (5)
Area under blood sugar-time curve following insulin (arbitrary units)	828 $\pm$ 187 (11)	1054 $\pm$ 241 (11)	842 $\pm$ 313 (5)
	Control	5	14
Half-life of blood sugar following intrav. inj. of glucose (min.)	18.7 $\pm$ 1.2 (9)	25.6 $\pm$ 2.4 (7)	15 $\pm$ 1.5 (5)

Values shown are mean $\pm$ stand. error of mean.

Figures in parentheses indicate No. of animals from which data derived.

sorption can as readily explain the facts. Furthermore, when hypertension and azotemia are present, oral glucose tests may show high prolonged curves similar to those of mild diabetes(11). Thus the evidence of a specific alteration in carbohydrate metabolism is not strong.

In our work, the animals had moderately severe nephrosis but were not moribund; they were studied early in the course of the disease, and again at a later date when their illness corresponded to chronic nephrosis without azotemia in humans.

In the first 4-7 days of nephrosis, Drabkin and Marsh observed a fall in blood sugar levels and in hepatic glycogen(2). At a comparable period in the present work, a decrease in post-absorptive glycemia was noted, together with an increased responsiveness to insulin and a decrease in glucose tolerance. None of these changes was marked and the latter 2 parameters soon returned to normal.

TABLE IV. Effects of Fasting in Experimental Nephrosis.

	Fast day 1		Fast day 2	
	Control	Nephrotic	Control	Nephrotic
Nitrogen excretion (mg/day)	160	172	148	132
Blood sugar* (mg/100 ml)	69	82	64	68
Protein excretion (% of mean excretion of preceding 5 days)		64		49
Wt loss (cumulative) (g)	10	8	21	18

* Blood taken on following morning.

Each figure represents mean value for 5 animals.

Hypoglycemia was not seen in the post-absorptive state at any time during the course of the disease or after a 48-hour fast carried out when the disease was "stabilized." This would indicate a normal capacity for gluconeogenesis.

The proteinuria in these animals, though high, accounted for only 2-3 calories/day, and after the first 5-10 days did not disturb the nitrogen balance. It is unlikely that such a small energy loss would disturb the homeostasis sufficiently to cause a significant decrease in gluconeogenesis or an increase in mobilization of depot fat great enough to produce hyperlipemia, as suggested by the concept of "protein diabetes."

Summary. Urinary glucose, blood glucose, insulin sensitivity and glucose tolerance were measured in experimentally nephrotic rats. In the early stage of nephrosis there was a slight fall in blood sugar concentration, a decrease in glucose tolerance and a decrease in insulin sensitivity. The latter 2 parameters later returned to normal. The response of nephrotic rats to fasting was essentially the same as that of normal rats. The grosser aspects of carbohydrate metabolism appear to be normal in experimental nephrosis.

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Received May 29, 1957. P.S.E.B.M., 1957, v95.

Multiplication of Foot and Mouth Disease Virus in Adult Kidney, and Embryonic Lung and Heart Bovine Tissue Cultures. (23333)

L. MELÉNDEZ V., A. GAGGERO C., R. RODRIGUEZ T., AND M. NORAMBUENA G.
(Introduced by J. F. Enders)

Animal Virus Department, Instituto Bacteriológico de Chile, Santiago

The multiplication of foot and mouth disease virus in tissue culture has been recently described by Seller(1), Bachrach(2), and Mazzaracchio(3) by using cultures of both bovine and pig kidney cells. The relatively large scale of virus production obtained with these methods seems to prove that tissue culture techniques are, presently, the most promising way for mass production of virus and a useful technic for the general assay of its activity. In our laboratory, we have succeeded in multiplying our 3 types of f. and m. virus, by using cultures of adult bovine kidney cells, and foetal bovine lung and heart tissues.

Methods. At the beginning we used the Dulbecco and Vogt method modified by Youngner(4), in slant tubes. After the work by Bodian(5) we started the setting up of Roux flasks of trypsinized cells with the advantage that its massive proliferation has also been used as source of cells for slant tubes. The cultures of bovine foetal lung and heart tissues have been made by planting minced tissue in plasma clot in Roux flasks. A good outgrowth of cells is obtained after 5 to 6 days in stationary incubation at 37°C. The nutrient medium for both types of cultures consisted in bicarbonated Hanks solution, with 0.5% lactalbumin hydrolysate, 20% horse serum and the usual antibiotics. Previously we had used the Enders' medium, but we changed due to our difficulties in obtaining regularly the cow amniotic fluid. The virus used has been our 3 types stock strains

of f. and m. virus. For the first virus inoculation, suspensions of cattle epithelium of fresh bovine passages were inoculated in the tubes. After 2 hours standing at 37°C the fluid was removed, and fresh nutrient medium was added. Ten passages were made of the 3 types of virus by using as inoculum a 10^{-8} virus dilution, before performing a T.C. titration of its activity.

Results. Two of the types of virus used gave the characteristic cytopathogenic picture at first passage. One strain of the O type required 2 blind passages before any cytopathogenic activity was detected. Virus was harvested in all cases at 18-20 hours after inoculation, and stored at -25°C, in aliquot parts with 10% peptone saline.

The virus multiplied in bovine kidney cells was titrated at the 10th passage in slant tubes of the same origin. Titres obtained showed that a definite multiplication has occurred in the cultures, as shown by titres of $10^{-6.5}$; $10^{-6.5}$; $10^{-5.5}$ and $10^{-6.5}$ ID/ml, for the strains C, C₂, A and O respectively.

The kidney cell cultures are now being used for typing field strains of virus and for titrations of guinea pig and bovine antisera.

Infected fluid from tissue culture has been used for the hyperimmunization of rabbits according to the method described by Ramos Alvarez and Sabin(6) and Freund and Thompson(7).

In the bovine foetal lung and heart tissue culture the same types of viruses have been

multiplied. The inoculum for this tissue has always been undiluted infected fluid from cultures of virus adapted to bovine kidney cells. The type of cytopathogenic damage in lung and heart tissue was different and apparently less severe than in kidney cells, and in no case a total destruction of the cell outgrowth was detected during 48 hours of observation. The great number of cells that remained unaltered at 12 hours, when a relatively high virus activity had been shown, led us to make several whole harvests of fluid at 6 hour intervals until 72 hours.

Each of these harvests was titrated in kidney cells, and their titers fluctuated around $10^{-6.5}$ and $10^{-4.5}$ ID/ml until 66 hours. Beyond this time the activity detected fell to 10^{-2} .

Virus of the C type from kidney cells, and heart and lung cultures has been titrated simultaneously in slant tubes, and in cattle by intralingual inoculation. No difference in titers was apparent in the 10-fold dilution method used in both titrations. The usefulness of the heart and lung tissue culture, as

a source of f. and m. virus for vaccination purposes is now being tested. A detailed investigation of the development of cytopathogenic activity of the f. and m. virus in bovine kidney cell cultures is now also under way.

Summary. Foot and mouth disease virus has been multiplied in bovine tissue cultures. When embryonic heart and lung bovine tissue cultures were inoculated with foot and mouth disease virus, it was possible to collect virus at 6 hour intervals until 66 hours without great decrease of virus activity.

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Received June 3, 1957. P.S.E.B.M., 1957, v95.

Potassium Acetate Inhibition of *Lactobacillus casei* and Its Reversal by Lithium, Sodium and Fatty Acids.* (23334)

M. N. CAMIEN AND M. S. DUNN

Chemical Laboratory, University of California, Los Angeles

In a previous communication from this laboratory it was reported that although *Lactobacillus casei* 280-16A fails to grow in conventional D- α -hydroxy acid-free media(1,2), its growth in the absence of a D- α -hydroxy fatty acid supplement may be promoted either by reducing the potassium content of the medium or by supplementing it with either lithium or (at considerably higher concentrations) sodium ion(3). Similarly, it was reported(3) that growth of *L. casei* 280-16B,

which normally does not require a D- α -hydroxy fatty acid supplement(4), is inhibited by potassium in D- α -hydroxy acid-free media, but not in D- α -hydroxy fatty acid-supplemented media, and that the inhibition may be reversed by either lithium or sodium. The parent strain of *L. casei* (strains 280-16A and 280-16B are mutants) was not inhibited by potassium under these conditions(3), presumably because it (but not the mutants) is able to elaborate D-lactic acid, which serves as an adequate D- α -hydroxy fatty acid source, but it has now been found that this organism is markedly inhibited by potassium when acetate is simultaneously supplied at elevated concentrations. This synergistic inhibition of *L.*

* Paper 119. This work was aided by grants from Natl. Multiple Sclerosis Soc., the U.S.P.H.S., and University of California. The authors are indebted to Evelyn Brown and Tibor Batonay for technical assistance.

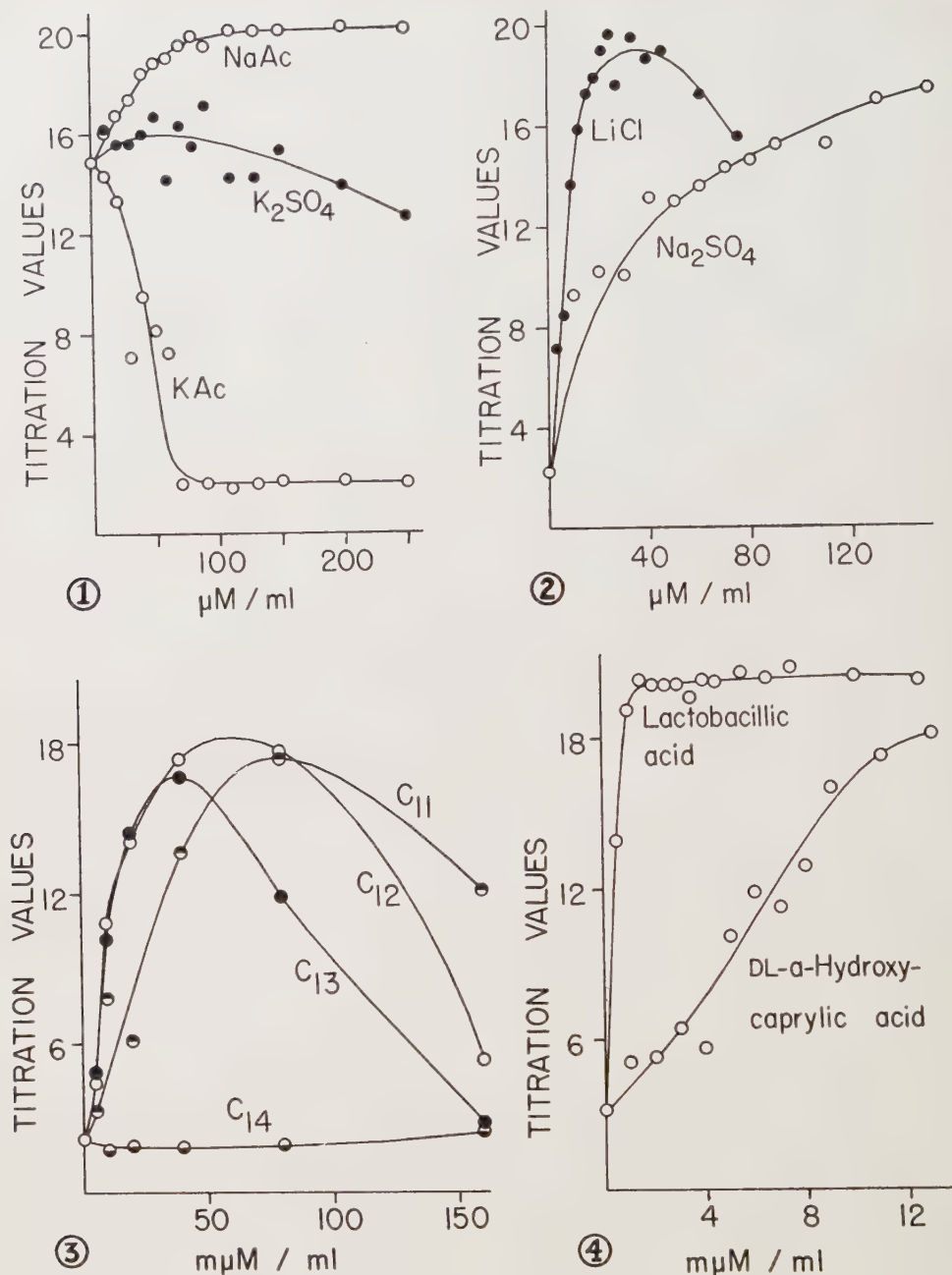


FIG. 1. Responses of *L. casei* to sodium acetate, potassium sulfate, and potassium acetate, respectively. Values on horizontal scale represent test concentrations of acetate and potassium ion (not potassium sulfate) in μ moles/ml. These concentrations are in addition to those initially present in the test medium (see *Methods*). Titration values (vertical scale) are calculated as ml of 0.01 *N* sodium hydroxide required to titrate 1 ml of test culture.

FIG. 2. Responses of *L. casei* to lithium chloride and sodium sulfate, respectively, in the test medium supplemented with 150 μ moles of potassium acetate/ml. Values on horizontal scale represent test concentrations of lithium and sodium ion (not sodium sulfate) in μ moles/ml. These concentrations are in addition to those initially present in test medium (see *Methods*). Titration values (vertical scale) are calculated as in Fig. 1.

FIG. 3. Responses of *L. casei* to hendecanoic (C_{11}), lauric (C_{12}), tridecanoic (C_{13}), and myristic (C_{14}) acids, respectively, under test conditions stipulated in Fig. 2. Test concentrations (horizontal scale) are given in $m\mu\text{mols/ml}$.

FIG. 4. Responses of *L. casei* to lactobacillic and DL- α -hydroxycaprylic acids, respectively, under test conditions stipulated in Fig. 2. Test concentrations (horizontal scale) are given in $m\mu\text{mols/ml}$.

casei by potassium and acetate is the subject of the present report.

Methods. *Lactobacillus casei* 7469 (American Type Culture Collection number) was employed, following essentially the experimental procedures described previously(1-4). The test medium contained casein digest† 0.6%, glucose 2%, vitamin-mineral mixture (6) 20.6 mg %, buffer solution‡ 5 ml %, and Tween 40§ 4 mg %. Its pH, before sterilization, was 6.5. This medium, when not supplemented additionally, provided potassium at 80 $\mu\text{mols/ml}$, acetate at 146 $\mu\text{mols/ml}$, and sodium at 9.5 $\mu\text{mols/ml}$. An additional 4.5 μmols of sodium/ml (final concentration) was included with the inoculum suspension, which was made up in sterile 0.8% sodium chloride solution. Lithium was not deliberately provided in the basal medium, but traces of this element were probably present as an impurity. The results to be discussed were obtained with an incubation period of 40 to 42 hours at 35°.

Results. Typical responses of *L. casei* to potassium and acetate are shown in Fig. 1. It may be seen (Fig. 1) that these agents together (as potassium acetate), but not separately, were markedly inhibitory, effecting 100% inhibition at 80 $\mu\text{mols/ml}$. Inhibition resulting with 150 μmols of potassium acetate/ml was completely reversed by lithium at 10 $\mu\text{mols/ml}$ (Fig. 2), by sodium at 85 $\mu\text{mols/ml}$ (Fig. 2), by hendecanoic, lauric and tridecanoic acids at 48, 25 and 23 $m\mu\text{mols/ml}$, respectively (Fig. 3), by DL- α -hydroxycaprylic acid at 8.8 $m\mu\text{mols/ml}$ (Fig.

4) and by lactobacillic acid|| at 0.6 $m\mu\text{mols/ml}$ (Fig. 4). The 18-carbon unsaturated acids, oleic, linoleic and linolenic acids, were very effective in reversing potassium acetate inhibition, their activities in this respect being approximately the same as that of lactobacillic acid (Fig. 4). Erucic, licanic and *trans*-vaccenic acids had activities of a lower order of magnitude approximating that of DL- α -hydroxycaprylic acid (Fig. 4). The percentage activities¶ of the straight chain DL- α -hydroxy fatty acids according to chain length and with the value of 100 arbitrarily assigned to DL- α -hydroxycaprylic acid (Fig. 4) were as follows: C_3 7, C_6 73, C_7 89, C_8 100, C_9 89, C_{10} 67, C_{11} 57, C_{12} 95, C_{13} 130, C_{14} 220, C_{15} 220, and C_{16} 130. The percentage activities of the straight chain non-hydroxylated saturated acids, on the same basis, were: C_{10} 5, C_{11} 20, C_{12} 50, C_{13} 50, C_{14} 0, C_{15} 0.

Discussion. These results strongly suggest that potassium acetate inhibits growth of *L. casei* 7469 by interfering with fatty acid biosynthesis, and it is curious in this regard that acetate, which is normally a fatty acid precursor, should under the present conditions assume the apparent role of an antimetabolite. The function of potassium as a co-inhibitor in these experiments is also apparently incongruous since this element is generally thought to be relatively non-toxic(8) to lactobacilli as compared to sodium and lithium, which promote growth under the present conditions. These considerations suggest that perhaps potassium and acetate do not inhibit directly, but rather effect the over-production of some normal metabolite, which at excessive

† Prepared from vitamin-free casein essentially as described by Roberts and Snell(5) except that hydrochloric acid was employed in place of acetic acid to neutralize the alkaline mixture after digestion and the charcoal treatments were omitted.

‡ Same as that described previously(6) except that sodium acetate was replaced by equivalent amount of potassium acetate.

§ Atlas Powder Co. product employed as a 10% stock solution in 50% ethanol.

|| A derivative of *L. casei* lipid(7). This material was kindly supplied by Professor Klaus Hofmann, Univ. of Pittsburgh School of Medicine.

¶ Calculated as 100 times the quotient of molar concentration of test acid effecting 50% reversal of inhibition divided by molar concentration of DL- α -hydroxycaprylic acid effecting 50% reversal of inhibition in a simultaneous test.

concentrations is able to inhibit growth. Myristic acid, for example, could be derived from acetate and is known to effect a marked inhibition of *L. casei* reversible by either lactobacillic acid or unsaturated fatty acids at relatively low concentrations(9). The effects of sodium and lithium according to this hypothesis would be to relieve the inhibition by impairing synthesis of the toxic product. It is entirely possible, on the other hand, that either sodium or preferably lithium activates an enzyme system concerned with essential fatty acid biosynthesis and that potassium inhibits the system by competition with sodium and lithium. The inhibitory effects of acetate in such a system might be due to competition of acetate with fatty acid intermediates.

Superficially the synergistic inhibition of *L. casei* 7469 by potassium and acetate appears to be closely related to the genetic block in *L. casei* strains 280-16A and 280-16B, since growth of the latter strains is promoted by either lithium or sodium(3) and by a variety of DL- α -hydroxy fatty acids(2). Further investigations are being undertaken to define

this possible relationship more exactly.

Summary. Growth of *L. casei* 7469 in a low-sodium, fatty acid-free medium is inhibited synergistically by potassium and acetate. Neither potassium nor acetate is significantly inhibitory when tested separately. The inhibition is readily reversed by either lithium or sodium and by a wide variety of fatty acids, the most effective of which are lactobacillic, oleic, linoleic, and linolenic acids.

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Received June 4, 1957. P.S.E.B.M., 1957, v95.

Action of Diphtheria Toxin on Cells Cultivated *in vitro*. (23335)

E. S. LENNOX* AND ALBERT S. KAPLAN (Introduced by L. M. Henderson)

Departments of Bacteriology and Chemistry, University of Illinois, Urbana

Tissue culture technics that have recently proved useful in quantitative virology can also be profitably applied to the study of some toxins. This note is a preliminary report of experiments on the action of diphtheria toxin on mammalian cells cultivated *in vitro*(1).†

Methods and materials. Most of the experiments were done with monolayer cultures of cells in glass tubes. Cultures of rabbit kid-

ney cells in tubes were prepared by the technic of Rappaport(2) and cultivated in Earle's saline containing 0.5% lactalbumen hydrolysate (Nutritional Biochemical) and 3% bovine serum. HeLa cells (strain obtained from Tuskegee Institute); 2 kinds of monkey kidney cells, a normal strain and several altered derivatives, from Dr. Raymond C. Parker (3); human epithelial carcinoma cells, HEP #2 from Dr. Alice Moore were grown in Earle's saline containing 0.5% lactalbumen hydrolysate, 0.1% yeast extract, and 20% horse serum. Additions of diphtheria toxin†

* Fellow of the National Foundation for Infantile Paralysis until June 1956.

† The authors gratefully acknowledge support from American Cancer Soc. (grant to Dr. S. E. Luria recommended by Committee on Growth), USPH Grant, and the the Univ. of Illinois Research Board.

‡ We are indebted to Eli Lilly and Co. for generous supply of diphtheria toxin and antitoxin.

TABLE I. Titration of Diphtheria Toxin in Tissue Culture.

Cells	Material inoc.	Cytopathic endpoint
		L_t
Rabbit kidney	Crude toxin	$1 \times 10^{-4} - 1 \times 10^{-5}$
Monkey "	" "	<i>Idem</i>
HeLa	" "	1×10^{-3}
HEP #2	" "	"
HeLa	Purified toxin	3×10^{-3}
Altered monkey kidney (6 strains)	Crude toxin	$> 1^*$
Rabbit kidney	Toxoid	$> 20^*$
	Boiled toxin	$> 1^*$
HeLa	Toxin and antitoxin	$> 0.1^*$

* No cellular changes were observed.

were made and the cultures were observed daily for cytologic changes. Titrations of toxin were carried out by adding 0.1 ml of serial ten-fold dilutions into the culture tubes containing 1 ml of medium. The cytopathic endpoint is taken as the highest dilution of toxin causing marked and progressive necrosis and detachment of the cells. Five or 6 tubes were used for each dilution.

Results. Rabbit kidney, HeLa, HEP, and normal monkey kidney cells were all susceptible to diphtheria toxin. Altered monkey kidney cells were not. With high concentrations of toxin, lysis of the sensitive cells occurred within 18 hours of addition of the toxin. With lower toxin concentrations more time elapsed before any change in the cells could be observed. Since no further changes could be observed beyond the fourth or fifth day, final readings were made on the fifth day. The results of several titrations are shown in Table I. The least amount of toxin that could be detected on rabbit kidney cells is about 10^{-4} - 10^{-5} L_t units.[§] Thus the method of assay as used here will not detect levels of toxin as low as detected in the rabbit intradermal inoculation test (5×10^{-6} - 10^{-6} L_t) (4).

[§] An L_t unit of toxin is defined as the volume in ml of toxin that gives most rapid flocculation with one standard unit of antitoxin. For our crude toxin, $1 L_t \cong 15$ minimum lethal doses for the guinea pig and the endpoint in the rabbit intradermal test is approximately 0.1 ml of a $1:5 \times 10^6$ dilution *i.e.* (2×10^{-6} L_t).

TABLE II. Effect of Time of Contact on Killing of Rabbit Kidney Cells.

Amt of toxin inoc., L_t /ml	Time of contact	Cytopathic reaction
1 or .1	~ 1 min.	—
	1 hr	+
	2 "	+
	3 "	+
	NR	+
.01	~ 1 min.	—
	1 hr	—
	2 "	+
	3 "	+
	NR	+
.001	~ 1 min.	—
	1 hr	—
	2 "	—
	3 "	—
	NR	+

NR = Toxin not removed.

+ = Necrosis of cells. — = No necrosis of cells.

HeLa and HEP#2 cells were somewhat less sensitive than rabbit or monkey kidney cells. There was a fairly good correlation between the endpoint found for HeLa cells with crude diphtheria toxin or with a highly purified preparation.||

That the reaction is specific is shown by the facts that a) toxoid, at a concentration 2×10^4 times greater than toxin, had no effect on rabbit kidney or HeLa cells; b) boiled toxin is not cytopathic; c) the action of the toxin is neutralized approximately to titer with specific antiserum; d) highly purified toxin is approximately equivalent to crude toxin (on the basis of L_t units) in cytopathic endpoint.

An experiment was performed to determine how long toxin must remain in contact with cells before its cytopathic action is no longer neutralizable by antitoxin. Cultures were inoculated with various dilutions of toxin which were allowed to remain in contact with the cells for varying lengths of time. The medium was then removed, the cell layers washed with fresh medium, and fresh medium containing antitoxin in large excess was put into the tubes. The results in Table II show that if 1 L_t unit of toxin were inoculated and allowed to remain for one hour the antitoxin could no longer suppress the cytopathic

|| The highly purified toxin was kindly supplied by Dr. M. Cohn.

TABLE III. Toxin Neutralization Test in Rabbit Kidney Cells.

Ratio of antitoxin : toxin	Toxin titer, L_t /ml	Cytopathic reaction
11.0	10^{-1}	—
4.5	10^{-2}	—
2.3	"	—
1.4	"	—
1.0	"	+
.5	10^{-1}	+

changes. The same was true with cultures inoculated with 0.1 L_t of toxin, the only difference being that cytopathic changes took longer to appear. For toxin at 10^{-3} L_t per ml a 3 hour contact caused no effects not overcome by the antitoxin. In separate experiments, toxin was found to be completely stable at 37°C in the medium without antitoxin for at least 48 hours. These experiments indicate that toxin can within one hour either become fixed to the cells and inaccessible to antibody or initiate the metabolic events leading to cell damage.

Toxin neutralization test can be readily done in tissue culture as the results of Table III show. In these experiments varying amounts of antitoxin were added to constant amounts of crude toxin and the mixtures were incubated at room temperature for 30 minutes before adding to the cultures for assay. As in the titrations described above, the period of observation was 5 days. These results indicate that toxin is neutralized when the ratio of antitoxin to toxin is slightly in excess of 1, roughly the same relationship as found in the skin test.

An assay of the MLD for 250 g guinea pigs of the crude toxin gave a value of approximately 15 MLD per L_t .

Discussion. These experiments, preliminary to a study of the mechanism of action of diphtheria toxin and to the use of toxin as a selective agent for cell variants, serve to il-

lustrate the relative ease and accuracy of using tissue culture in immunological research. Tissue culture may provide a system less complicated than the intact animal for elucidating the mechanism of diphtherial toxicity. In regard to the use of toxin resistance as a genetic marker, it should be noted that a group of strains—altered monkey kidney lines—can be distinguished from the parent line by resistance to high levels of diphtheria toxin. The resistance of these strains is not due to extracellular destruction of the toxin. This was ascertained by removing medium containing toxin that had been over a layer of altered monkey cells for as long as 8 days and assaying on rabbit kidney cells. The toxin titer was undiminished and was reversible by antitoxin.

Summary. Several types of mammalian cells which can be cultivated *in vitro* are susceptible to low concentration of diphtheria toxin. These cell lines may, therefore, be used as an assay system for diphtheria toxin and for antitoxin. There are altered cell lines, derived from sensitive ones which have lost their sensitivity to even very high levels of toxin. Diphtheria toxin resistance is then a possible genetic marker for study of cells in tissue culture.

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Received June 6, 1957. P.S.E.B.M., 1957, v95.

Distribution of Oxytocin, Vasopressin and Intermedin in Hypophysis of the Beaver.* (23336)

F. O. KELSEY,[†] L. SORENSEN, A. A. HAGEN AND H. J. CLAUSEN

*Departments of Physiology and Pharmacology and of Anatomy, State University of S. Dakota
School of Medicine, Vermillion*

The hypophysis of the beaver (*Castor canadensis*) has been found to lack an anatomically discrete pars intermedia.[‡] In this respect, the gland resembles that of the armadillo, whale, porpoise, manatee and fowl. In these latter species, the pressor, antidiuretic and the oxytocic activities are confined to neural lobe extracts while melanophore-expanding activity is confined to anterior lobe extracts (1-4). Furthermore, assays of the neural lobes of certain whales and armadillo showed a marked disproportion in the pressor-oxytocic ratio compared with that of the U.S.P. standard pituitary powder. The present study was undertaken to determine distribution of hormones in the beaver hypophysis, and to determine pressor-oxytocic and pressor-antidiuretic ratios in this species.

Materials and methods. Live beavers secured by trapping through cooperation of the South Dakota Department of Game, Fish, and Parks, were anesthetized by ether or sodium pentobarbital and decapitated. The pituitary gland was removed, weighed (Table I) and placed in acetone after separation into anterior and neural lobes and stalk. Portions of cortex and hypothalamus were also fixed in acetone. For assay purposes, the tissues were dried, ground finely and extracted with 0.25% acetic acid, 1 cc/mg of dried powder. Weights of the separated lobes are shown in Table I. Three lots of extracts were prepared; in 2 of these, 2 or 3 glands were pooled. Oxytocic activity was measured by U.S.P. method, which utilizes the vasodepressor effect in the anesthetized fowl. Antidiuretic activity was determined by Ham and Landis' modification of Burns' method which uses un-

anesthetized, hydrated rats (5). Pressor activity was assayed by pressor response of anesthetized cats. Melanophore-expanding activity was determined using hypophysectomized frogs. The U.S.P. Posterior Pituitary Reference Standard, which contains 0.4 oxytocic units/mg, was the standard used for oxytocic, pressor and antidiuretic activity.

Results. Oxytocic activity: The neural lobe of the beaver contained approximately 2 units of oxytocic activity/mg of dried powder, and the stalk approximately 0.4 unit/mg. No oxytocic activity could be detected in extracts of hypothalamus, cortex, or anterior lobe when administered in amounts equivalent to 10-fold the amount of neural lobe extract necessary to produce a definite effect.

Pressor activity: The neural lobe contained approximately 2 units/mg of dried powder and the stalk approximately 0.4 unit. Anterior lobe, hypothalamus and cortex failed to evoke a pressor response in amounts 10-fold greater than the effective dose of neural lobe extract.

Antidiuretic effect: An appreciable antidiuretic effect was observed when rats were given 0.6 μ g of neural lobe extract/100 g body weight. A comparable effect was obtained with a dose of 1.25 milliunits of standard/100 g. A significantly greater effect was noted with a dose of 5 μ g of beaver neural lobe/100 g, the effect being similar to that produced by the standard preparation in a dose of 10 milliunits/100 g. Thus, antidiuretic assays indicate activity of approximately 2 units/mg dried powder for beaver neural lobe. No antidiuretic effect could be elicited by extracts of anterior lobe, cortex or hypothalamus when given in amounts of 50 μ g/100 g rat.

Melanophore hormone: Appreciable darkening of hypophysectomized frogs occurred when 0.001 mg of anterior pituitary was injected in 0.5 cc saline in the ventral lymph

* A portion of this report was presented at meeting of Fed. Am. Soc. for Exp. Biol., Chicago, April, 1957.

[†] Studies carried out during the tenure of a Lederle Medical Faculty Award.

[‡] Clausen, H. J., and Kelsey, F. O.,

TABLE I. Wet Weight in Milligrams of Separated Portions of Beaver Hypophyses.

Date of collection	Body wt, kg	Sex	Wt of separated portions (mg)	
			Ant. lobe	Neural lobe + stalk
6/55	41.8	♂	97	25
2/56	37.4	♀	67	43
"	35.2	♂	83	30
"	37.4	♀	87	35
3/56	41.8	♂	80	49
6/56	41.8	♂	90	40

sac. A slight response could be detected with 0.5 mg of neural lobe and an equivocal response with 0.125 mg. An equivocal response was also obtained with 0.5 mg of hypothalamus. No melanophore expansion was noted with 0.5 mg of cortex.

Discussion. The results indicate that the neural lobe of the beaver possesses oxytocic, pressor and antidiuretic activity but contains only traces of melanophore-expanding activity. The pressor:oxytocic ratio is similar to that of the standard powder. Hagen[§] has found a similar ratio in freshly collected beef and sheep glands but a pressor-oxytocic ratio of 0.6 in the hog. Using different assay procedures, Stoepel(6) and Van Dyke *et al.*(7) have also found a pressor-oxytocic activity of less than 1 in this species. Earlier studies have indicated a pressor-oxytocic ratio of 1 in the manatee, porpoise, and chicken and of from 3 to 10 in the armadillo and whale. However, different U.S.P. standards were used in these 2 series of comparative assays and it cannot be assumed that the pressor-oxytocic ratio of the standard has remained constant since official assays have been confined to determination of oxytocic activity. The fact that the pressor-antidiuretic ratio was similar to that of the standard is in accord with the generally accepted viewpoint

[§] Hagen, A. A., from dissertation presented in partial fulfillment of requirements for degree of Master of Science, State Univ. of S. Dakota.

that the 2 activities are vested in the same moiety(8). Failure to demonstrate pressor, oxytocic and antidiuretic activity in extracts of hypothalamus was not considered significant since the assay procedures used are not very sensitive and no effort was made to dissect out the hypothalamic nuclei where these activities have been reported to be concentrated(9).

The presence of melanophore-expanding activity almost exclusively in anterior lobe extracts is in keeping with previous reports that this hormone is elaborated in the anterior lobe of these species in which a discrete intermediate lobe is lacking.

Conclusions. 1) The neural lobe of the beaver pituitary contains approximately 2 units of oxytocic, pressor and antidiuretic activity/mg dry weight. The stalk contains about 0.4 unit of pressor and oxytocic activity/mg. 2) The pressor-oxytocic and pressor-antidiuretic ratios of neural lobe extracts were similar to those of the U.S.P. standard pituitary powder. 3) The anterior lobe contains appreciable melanophore-expanding activity but no oxytocic, pressor or antidiuretic activity.

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Received June 7, 1957. P.S.E.B.M., 1957, v95.

Means of Increasing the Tolerated Dose of Isoniazid in Mice.

III. Certain Vitamins. (23337)

BENJAMIN PRESCOTT, GLADYS KAUFFMANN, AND WALTER D. JAMES
(Introduced by R. J. Huebner)

*U.S.P.H.S., Department of Health, Nat. Inst. of Allergy and Infectious Diseases, N.I.H.,
Bethesda, Md.*

In efforts to reduce toxic reactions caused by use of isoniazid (INH) in therapy of tuberculosis, it has become increasingly evident that this chemical alters normal metabolism and may be "detoxified" or transformed by supplemental use of substances essential to normal enzymic processes. Thus it has already been demonstrated that various amino acids, in particular glycine supplemented with sodium glucuronate(1) and cysteine(2), or certain alpha keto acids(3), or cycloserine(4), or glycerine(5) or propylene glycol(3) as solvents, are severally effective in increasing the tolerated dose of INH administered orally in DBA strain of mice. Hence, it seems logical that similar biotransformation may also occur if INH is supplemented with vitamins. The present report gives results of an investigation of the effect of certain vitamins on acute and chronic toxicity of INH in DBA mice.

Methods. In this strain of mice, the amount of INH which is 100% lethal by oral administration is 6 mg/20 g mouse (300 mg/kg). Consequently, the effectiveness of the various vitamins was tested initially with a somewhat larger dose, 8 mg, of isoniazid. The procedure consisted of mixing in a test tube the required amount of INH and of vitamin in water to such volume that the desired concentrations were contained in 0.5 ml for oral administration. When necessary, the hydrogen ion concentration was adjusted to pH 5. Ergosterol required mineral oil rather than water for suspension.

Results. *Effect of certain vitamins on acute oral toxicity of isoniazid in mice.* Initial tests with 8 mg INH/20 g mouse and an equimolar concentration of each of 15 vitamins are summarized in Table I in the order of effectiveness. With 3 others and the isomeric isonicotinic acid, a 5:1 molar ratio was used. On the assumption that, as food accessory substances, only minute amounts of vitamins are

necessary, smaller amounts of these 4 were tested but were much reduced in their activity as detoxicants. It is possible that those with little or no effect might be active in other concentrations, except that with increased amounts some of the vitamins, such as menadione or choline, are toxic *per se*. Also in this Table are subsequent tests with other concentrations of the 4 vitamins, pyridoxal-HCl, pyridoxine-HCl, p-aminobenzoic acid, and nicotinic acid, which were better than 50% effective in increasing survival rate of the mice. Pyridoxal-HCl is outstanding in that doses ranging from 8 to 20 mg/mouse protected all but one of the animals against this lethal 8 mg dose of INH. This vitamin was therefore selected for a more thorough study.

Effect of repeated administrations. Various concentrations of INH were administered by gavage at 24 and 72 hour intervals with pyridoxal-HCl in molar equivalent proportions; and also one test was made of 8 mg INH with 8 mg pyridoxal, *i.e.*, a 1:0.66 molar ratio. As seen in Table II, this latter mixture prolonged survival time better than the others administered daily. Indeed it was comparable to the most effective mixture given at 72-hour intervals, namely 20 mg INH with 30 mg pyridoxal. This result may have been due to the fact that the INH and pyridoxal formed a hydrazone in the test tube before administration which was only gradually hydrolyzed *in vivo*. Thus in the mixture of 8 mg INH and 8 mg pyridoxal, which contained such a hydrazone, the amount of INH gradually liberated would theoretically be 5.3 mg. During this gradual hydrolysis, some INH was being excreted. At the same time the free INH (2.7 mg) of the original mixture was also gradually eliminated. Accordingly, at no time would there be a toxic dose. This is demonstrated by the fact that up to 8 daily doses were administered without lethal effect.

TABLE I. Effect of Certain Vitamins on Acute Oral Toxicity of Isoniazid in DBA Mice. 8 mg INH

Vitamin	Vit. amt, mg*	No. surviving/No. tested	% survival
Pyridoxal-HCl	8	10/10	100
"	12*	16/16	100
"	15	10/10	100
"	20	9/10	90
"	30	6/10	60
Pyridoxine-HCl	60†	16/20	80
"	30	2/10	20
P-Aminobenzoic acid	12	6/10	60
"	8*	15/26	58
Nicotinic acid	36†	24/46	52
"	25	3/10	30
"	7*	2/20	10
Nicotinamide	36†	7/15	47
Menadione	10	7/16	44
[Isonicotinic acid]	36†	16/46	35
Alpha-tocopherol	25	3/10	30
Choline-HCl	8	1/6	17
Folic acid	25	1/6	17
Ergosterol	23	2/17	12
Pyridoxamine-HCl	14	0/6	0
Ascorbic acid	10	0/6	0
Biotin	14	0/10	0
Riboflavin	20	0/10	0
Ca pantothenate	28	0/6	0
Vitamin A	17	0/10	0
Thiamine-HCl	20	0/20	0
Inositol	13	0/20	0

* Indicates equimolar ratio.

† Indicates 5 millimols vitamin/millimol INH.

In miscellaneous low titer group, all are equimolar unless otherwise marked.

Only then was there evidence of possible accumulation in the tissues. The combination of 20 mg INH and 30 mg pyridoxal given at 72-hour intervals was of special interest, inasmuch as in these amounts each chemical alone had been found lethal within an hour.

Yet this hydrazone was administered twice weekly for 8 doses before the 50% mortality endpoint was reached. From these tests it may be concluded that concomitant pyridoxal permits mice to tolerate repeated administration of as much as 3 times the lethal dose of INH for several weeks. In addition, a test of repeated administrations of each of 2 other vitamins, nicotinic acid and p-aminobenzoic acid, with 8 mg INH at 72 hour intervals is included in Table II. Of the 2, nicotinic acid was slightly superior, but in view of the fact that a 5:1 molar ratio was necessary, it was not as effective/mole as pyridoxal.

Blood levels. Assay of INH blood plasma levels in DBA mice at various time intervals after administration of INH-pyridoxal mixtures was carried out by the "pyridyl" method (6). The mixture of 8 mg INH and 12 mg pyridoxal (molar equivalents), *i.e.*, the hydrazone, gave a constant level of approximately 1 mg% for 7 hours. This lower level may indicate the extent to which the hydrazone, formed in the test tube before administration, has been hydrolyzed *in vivo* during the first hour after administration. Also purified hydrazone (20 mg) in one test gave a constant level of approximately 1 mg% for 7 hours. However, the one-hour level after an injection of a mixture of 8 mg INH with 8 mg pyridoxal was 4.2 mg%. This higher level may be accounted for by the amount of free INH in solution (2.7 mg) in excess of that used in forming the hydrazone. For it was found that the plasma level assayed 1 hour after a 2 mg oral dose of INH was 3.4 mg%. It remains to be seen whether a low but persis-

TABLE II. Effect of Repeated Oral Administrations of Certain Vitamin-Isoniazid Mixtures in DBA Mice.

Isoniazid, mg	—Vitamin— mg	Time intervals, hr*	No. of mice	No. mice surviving the following No. of doses											
				1	2	3	4	5	6	7	8	9	10	11	12
8	Pyridoxal-HCl	8	24	10	10	10	10	10	10	10	10	6	4	4	3
15	"	22.5	24	9	9	5	2								2
20	"	30	24	30	25	24	23	9							
8	"	12	72	16	16	15	13	12	12	10	9	3			
10	"	15	72	9	8	8	7	4	3	3					
20	"	30	72	20	20	20	20	17	14	14	11	9	6		
8	Nicotinic acid	36	72	16	14	12	10	10	10	10	9	7	7	4	
8	p-Aminobenzoic acid	8	72	10	8	8	7	7	7	5	5	0			

* 24 hr indicates daily, Monday through Friday inclusive; 72 hr, twice weekly, Monday and Thursday.

tent INH blood level is more efficacious in treatment of human tuberculosis than an early higher peak followed by rapid decrease in INH concentration in the blood stream.

Effect of certain vitamins on INH inhibition of Mycobacterium tuberculosis. One method has been applied to determine the effect of some of these vitamins on the bacteriostatic action of INH on one avirulent strain of *Mycobacterium* B103. The test was carried out by a procedure previously reported (1). The vitamins, tested in molar equivalent amounts, included: pyridoxal-HCl, pyridoxine-HCl, p-aminobenzoic acid, nicotinic acid and its isomer isonicotinic acid, nicotinamide, and menadione, and for comparison the purified hydrazone of INH and pyridoxal. Of themselves none of the vitamins except possibly menadione demonstrated any inhibitory action on growth for the period of observation. When mixed with INH, there was questionable interference by nicotinamide, but with the others no antagonism to the bacteriostatic action of INH. In contrast, the purified hydrazone by itself was as effective as INH in inhibiting growth, and in mixture with INH did not interfere with its activity as a tuberculostatic agent.

Discussion. Interest in the use of vitamins with INH has been stimulated by observations of many clinicians that toxic manifestations in 40% of tuberculous patients under treatment with INH include peripheral neuritis and other symptoms similar to those observed in pyridoxine deficiency. In applying Zeller's observation(7) that toxicity of INH is largely due to inactivation of amine oxidases, Reilly and collaborators(8) demonstrated that the INH toxicity could be partially reversed in mice by use of small amounts of pyridoxine. Similarly Biehl and Vilter(9) concluded that simultaneous pyridoxine prevented development of neuritis, and from metabolic studies suggested that INH inactivated amino acid decarboxylase by virtue of forming a hydrazone with the essential coenzyme, pyridoxal phosphate. Without discussion of mechanism, Tirunarayanan and Vischer(10) reported that among 6 B vitamins studied as possible detoxifying agents for INH in guinea pigs, only pyridoxine was

effective. It is pertinent that pyridoxine and pyridoxal normally have similar activity *in vivo*. Accordingly, it may be that depletion of pyridoxal from tissues, which occurs with INH alone, would be prevented by the use of the pyridoxal isonicotinyl hydrazone.

It is of interest that absorption of INH may be more prolonged due to gradual hydrolysis of the hydrazone, and hence effectiveness without toxic manifestations may be extended, as suggested from the constant lower blood level.

It should be noted that our report includes study of doses of INH ranging from 8 to 20 mg/mouse, all of which are well above the 6 mg lethal dose. The implication may be that pyridoxal used with sublethal doses of INH would reduce or even eliminate some of the toxic manifestations observed in human tuberculous patients under treatment with this chemotherapeutic agent.

Summary. Certain vitamins have been found effective in increasing the tolerated oral dose of isoniazid (INH) in DBA mice. Four of the group, pyridoxal-HCl, pyridoxine-HCl, p-aminobenzoic acid, and nicotinic acid, permitted 50% or more, mouse survival in acute toxicity tests with 8 mg INH (400 mg/kg). Only pyridoxal-HCl, in 1- or 2-molar equivalent concentration, protected 100% of mice against this dose. This vitamin in appropriate concentration formed a hydrazone with INH and the compound containing as much as 20 mg INH/mouse was tolerated for 8 semi-weekly administrations. Blood plasma levels of INH following administration of the hydrazone were somewhat lower than those after a sublethal dose of INH alone, but were constant and more prolonged, presumably due to gradual *in vivo* hydrolysis of the hydrazone. Six vitamins, as tested, did not interfere with *in vitro* bacteriostatic action of INH on one avirulent strain of *Mycobacterium*, but the pyridoxal isonicotinyl hydrazone *per se* was as effective as INH alone as a tuberculostatic agent.

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Received June 12, 1957. P.S.E.B.M., 1957, v95.

Endogenous Respiration of *Serratia marcescens* and Oxidation of Added Carbohydrates.* (23338)

AARON E. WASSERMAN AND WILLIAM J. HOPKINS (Introduced by Joseph S. Gots)

Eastern Regional Research Laboratory, Agricultural Research Service, U. S. Dept. of Agriculture, Philadelphia, Pa.

During an investigation of the oxidative patterns of *Serratia marcescens*, cells grown in one of several media consistently yielded an anomalous pattern of substrate oxidation when the data were corrected for endogenous metabolism in the classical manner. Subsequent study of the data indicated that this unusual behavior may be due to suppression of the endogenous metabolism of the organisms. The role of endogenous metabolism during oxidative dissimilation of various substrates has been variously reported. Production of $C^{14}O_2$ from C^{14} "labeled" *Streptomyces coelicolor* was found to be the same during oxidation of glucose and pyruvate as it was in endogenous metabolism(1). Wilner and Clifton(2) reported that the endogenous respiration of a strain of *Bacillus subtilis* was not suppressed during exogenous respiration, and that correction for the former was necessary to obtain satisfactory equations for carbon assimilation. Conversely, endogenous respiration of *Pseudomonas saccharophila* was reported to be inhibited during oxidation of assimilable substrates(3,4), and the oxidation data for various carbohydrates by *Prototheca zopfii* were more amenable to analysis if the endogenous respiration of the organisms was disregarded(5). The present report suggests that the conflicting conclusions of the cited

studies may be a result of different growth conditions, in addition to the differences related to species specificity.

Methods. *S. marcescens*[†] was grown in media containing 5% skim milk solids, 3% Armour's peptone,[‡] and 2% of one of the following carbohydrates. *Neutral glucose*: glucose added to medium prior to sterilization at 121°C for 15 minutes. The pH was 7.0-7.2 without adjustment. *Neutral gluconate*: gluconic acid was dissolved in a few ml water and adjusted to pH 6.0 prior to addition to the medium. Following sterilization the pH dropped to approximately 4.0. Sterile NaOH was added to raise the pH to approximately 6.0. *Acid gluconate*: gluconic acid was prepared as above and added to the medium. However, after sterilization the pH was allowed to remain at pH 4.0. After 16 hours growth on a reciprocal shaker at 30°C, the cells were harvested by centrifugation, washed once with water, and stored at 5°C until used. The Warburg experiments were carried out by conventional manometric technics. Gluconic acid was obtained as glucono-delta lactone from the Eastern Chemical Corporation.

[†] Obtained thru the courtesy of Dr. R. G. Benedict, Northern Utilization Research and Development Division, Agricultural Research Service, U. S. Dept. of Agriculture.

[‡] Mention of commercial products does not imply endorsement by the U. S. Dept. of Agriculture over similar products not mentioned.

* This work was performed under fund supplied by The Chemical Corps, Fort Detrick, Frederick, Md.

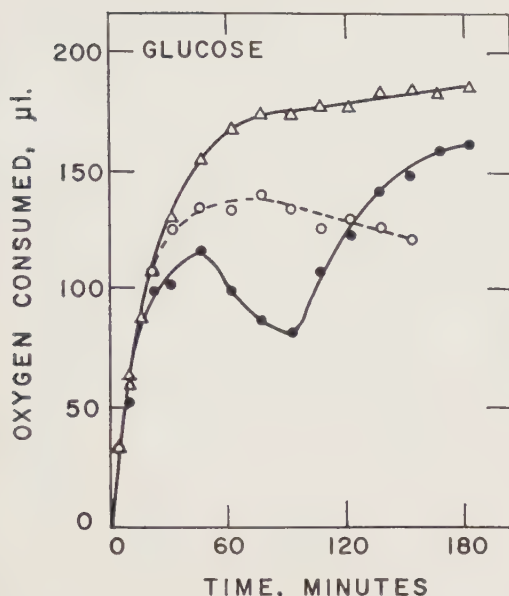


FIG. 1. Glucose oxidation by *S. marcescens* harvested from several media. Each flask contained 0.5 ml 0.05 *M* tris (hydroxymethyl) amino methane buffer, pH 7.2; 100 μ g inorganic phosphorus; 4 mg (dry wt) appropriate cells; 2.5 μ M glucose; water to 2.0 ml. Temperature -30°C . Δ — Δ Neutral glucose-grown cells; $\circ$ — $\circ$ Neutral gluconate-grown cells; $\bullet$ — $\bullet$ Acid gluconate-grown cells.

Results. Representative oxygen consumption curves of glucose dissimilation by cells grown on the 3 media were obtained in the classical manner by subtracting endogenous respiration of the cells from the total respiration. Cells harvested from the glucose and neutral-gluconate media oxidized glucose in a manner described by the curves in Fig. 1. The acid gluconate-grown cells, however, produced a minimum in the curve (Fig. 1). This minimum is due to the fact that the rate of endogenous metabolism of the cells was greater than the rate of metabolism in the presence of glucose. Inspection of the endogenous metabolic patterns of the 3 cell preparations showed that respiratory activity of the glucose-grown cells was qualitatively similar to that of the neutral gluconate-grown cells, but that of the acid gluconate-grown cells was considerably higher and varied in rate.

The anomalous pattern of oxidation of the acid gluconate-grown cells in the presence of

substrate could not be exclusively attributed to increased endogenous metabolism. Correction for the high endogenous respiration should yield normal curves as obtained with the other cell preparations. The pattern of respiration was analyzed by comparing rate of oxygen consumption by neutral gluconate- and acid gluconate-grown cells in the presence and absence of glucose (Table I).

Endogenous utilization of oxygen by the neutral gluconate-grown cells proceeded at a low, fairly uniform rate. In the presence of glucose, oxygen consumption was regular, but at an increased rate, then dropped to that of the endogenous cells. However, the acid gluconate-grown cells showed a sharp increase in endogenous rate of oxygen consumption after 30 minutes, which was maintained for approximately an additional 60 minutes before returning to the original lower level. In the presence of glucose, initial oxygen uptake quickly attained a higher rate, which was maintained for approximately 135 minutes before returning to the endogenous rate. The increase in endogenous metabolic rate observed between 30 and 90 minutes was not reflected in the respiratory pattern of the cells in the presence of glucose.

It was also observed that the course of

TABLE I. Course of Oxygen Consumption in Presence and Absence of Glucose by Acid Gluconate- and Neutral Gluconate-Grown *S. marcescens*.

Time, min.	Neutral grown cells		Acid grown cells	
	Endog.	Glucose	Endog.	Glucose
5	0*	29*	5*	34*
10	7	36	16	35
15	8	32	14	47
20	7	28	17	31
30	9	27	36	38
45	16	25	66	81†
60	8	7	70	52
75	10	17	59	47
90	14	8	52	47
105	17	9	33	58
120	0	4	31	45
135	10	6	25	43
150	10	6	22	29
165	—	—	20	31
180	—	—	17	19

* The figures represent μL oxygen consumed in each time interval.

† This high reading may be due to experimental error since subsequent experiments maintained a constant rate of glucose oxidation.

dissimilation of gluconic acid by the 3 cell preparations was similar to that of glucose. Acid gluconate-grown cells also oxidized 6-phosphogluconate and fructose 1,6-diphosphate in a pattern that varied from that of the other two cell preparations. The observed differences in the response of the cell preparations is not due to the pH of the growth medium alone, since cells grown on acidified (pH 4.0) glucose medium oxidized the various substrates in a manner similar to that of the neutral glucose-grown cells.

Summary. Endogenous oxygen consumption of *Serratia marcescens*, grown on a medium containing glucose at pH 4 and 7 or gluconate at pH 6, continues unchanged during oxidation of glucose and several other substrates. Correcting total oxygen consumption in the presence of a substrate for the endogenous oxygen consumption, affords an accurate representation of the course of oxidation of the substrates. *S. marcescens* grown in glu-

conate-containing medium at pH 4 has a higher endogenous oxygen consumption than cells grown on the other media. At one stage of the endogenous metabolism a sharp increase in rate of oxygen consumption occurs. Such sharp increase in oxygen consumption does not occur during oxidation of glucose and several other substrates. Hence correction of total oxygen consumption in the presence of the substrates for endogenous respiration of the cells may lead to an erroneous interpretation of the data.

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Received June 11, 1957. P.S.E.B.M., 1957, v95.

Inhibition of Prothrombin Activation in Sodium Citrate Solutions.* (23339)

WALTER H. SEEGER AND RICARDO H. LANDABURU

Department of Physiology and Pharmacology, Wayne State University College of Medicine, Detroit, Mich.

In 25% sodium citrate solution purified prothrombin slowly transforms to thrombin (1). The discovery of this transformation cleared the view that prothrombin, as a single molecular entity, contains all of the structural material required in the thrombin molecule (2). The activation is autocatalytic and is initiated or accelerated by the addition of thrombin. This fundamental consideration alerts us to the possibility that prothrombin activation under physiological conditions may also be autocatalytic, that thrombin may be the only enzyme in prothrombin activation and that other factors are accessory.

The autocatalytic activation of purified prothrombin in sodium citrate solution can be accelerated with an organic compound of

known structure such as 3-chloro-4,4'-diaminodiphenyl sulfone(1). By substituting an amino group for the chlorine atom and thus having 3,4,4'-triaminodiphenyl sulfone the autocatalytic activation is inhibited. In a similar way soybean trypsin inhibitor stops the autocatalytic process(3). We thus have a greatly simplified view of prothrombin activation that may actually be giving us an accurate concept of the minimal chemical requirements for prothrombin activation and its inhibition. The inhibitors 3,4,4'-triaminodiphenyl sulfone and soybean trypsin inhibitor are however not to be anticipated in plasma or serum but apparently no one has inquired whether other inhibitors of prothrombin activation in sodium citrate solution might be found in plasma or serum. With this idea in mind we conducted many experiments and

* This investigation was supported by research grant from N.I.H., U.S. Public Health Service.

have found that plasma and serum do indeed function as an inhibitor even if considerably diluted. At a 12-fold dilution of serum the autocatalytic transformation of prothrombin to thrombin can virtually be reduced to zero. To a certain extent this property of serum is adsorbed on barium carbonate.

Methods. Purified prothrombin was prepared from bovine plasma as described by Seegers and Alkjaersig(4). These prothrombin preparations were also used to obtain purified biotrombin by the methods described by Seegers and Alkjaersig(4). Prothrombin activity and thrombin activity were measured as previously described(5,6). Barium carbonate eluate is a preparation obtained from bovine serum as described by Seegers, Johnson, Fell and Alkerjaersig(7). In principle, barium carbonate is added to serum, removed by centrifugation, washed and mixed with a 5% sodium citrate solution. This elutes protein which can be fractionated with ammonium sulfate. After dialyzing the protein is obtained by drying from the frozen state. We suppose this preparation to be rich in Factor VII (SPCA, convertin, cothromboplastin, stable conversion factor, β -thromboplastin), platelet cofactor II (PTC, Christmas factor) and other factors. Whatever this preparation may contain it has only been characterized accurately by a description of its preparation and the specific conditions under which it exhibits activity. As previously, we use the term barium carbonate eluate when referring to this preparation. Bovine serum was shaken with an equal volume of ether for 3 minutes. The ether was removed and the process repeated twice. This removes much of the antithrombin activity.

Results. Our observations are considered in terms of 3 experiments all performed several times and with variations. In one of these a solution of purified prothrombin was mixed with a solution of thrombin so that the final concentration was 3,000 and 240 units/ml respectively. Immediately sodium citrate was added to give a 25% solution. At room temperature thrombin activity increased during a period of 12 hours with a final yield of about 66% of the potential thrombin that could theoretically be expected from the

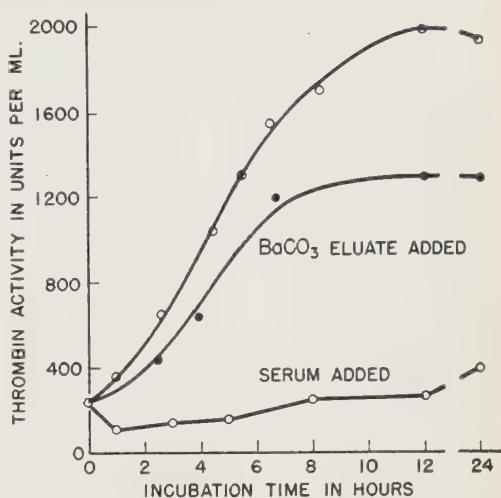


FIG. 1. Activation of purified prothrombin in 25% sodium citrate solution, and its inhibition with serum diluted 1:12 or barium carbonate eluate at a concentration of 2.5 mg/ml.

original prothrombin added (Fig. 1). The autocatalytic activation of prothrombin was typical of that previously reported.

In a second experiment bovine serum was diluted 12-fold and the amount of prothrombin, thrombin, and sodium citrate added was the same as in the previous experiment. We observed that the thrombin concentration in the mixture actually dropped during the first hour but then increased slowly during the next 24 hours. At most about 10% of the prothrombin eventually was converted to thrombin (Fig. 1). Serum possesses inhibitory properties. We also found that serum adsorbed with barium carbonate, as in the preparation described for barium carbonate eluate(7) still possessed the power to inhibit the formation of thrombin. The same was observed when barium sulfate was used in place of barium carbonate, but in either case we made no attempt to see whether more barium carbonate or sulfate would diminish the power of serum as an inhibitor.

In a third experiment we wanted to see whether prothrombin activation would be retarded with material adsorbed on barium carbonate and eluted with sodium citrate. For this purpose prothrombin and thrombin were again mixed in 25% sodium citrate solution just as in the first 2 experiments. The dry barium carbonate eluate was then added to

give a concentration of 2.5 mg/ml. Prothrombin was not converted to thrombin quite as rapidly as without the barium carbonate eluate and the thrombin yield was only 43% (Fig. 1). The eluate was an inhibitor of the interactions. To obtain even less thrombin more of the barium carbonate eluate could be added. Similar properties were found in another eluate prepared with the use of barium sulfate instead of barium carbonate. Seitz filtration of the barium carbonate eluate did not destroy or remove the hypothetical inhibitor. As another variant in our experiments the barium carbonate eluate was fractionated. For this purpose it was dissolved in water. The pH was brought to 5.4 and a precipitate formed which was removed. The supernatant solution was further acidified to pH 4.6. Another precipitate formed just as occurs in the final fractionation steps in the purification of prothrombin(4). It was taken up in water, neutralized and dried from the frozen state. This dry precipitate used in the same quantities as the original barium carbonate eluate inhibited the interactions to the same extent as the original barium carbonate eluate. We further determined that an active barium carbonate eluate could be made from plasma just as well as from serum.

Discussion. The autocatalytic activation of prothrombin in 25% sodium citrate solution is not only inhibited with 3,4,4'-triaminodiphenyl sulfone or soybean trypsin inhibitor but also by plasma, serum and protein adsorbed on barium carbonate. We postulate that there is at least one inhibitor in plasma and serum that can account for our experimental results. Although we find the adsorbed serum still possesses the hypothetical substance(s) we do not know whether this is on the basis that more barium carbonate might adsorb all or whether the material not adsorbed is qualitatively different from that which is adsorbed.

In our purification of prothrombin the inhibitor(s) is evidently not concentrated with the prothrombin otherwise it would not be possible to obtain thrombin in 25% sodium citrate solution. During the purification there are several possibilities for the elimination of the inhibitor(s) including that adsorp-

tion on magnesium hydroxide may not occur. On the other hand it is likely that the adsorption on barium sulfate or barium carbonate in the purification of prothrombin and Factor VII as described for example in the work of Duckert, Koller and Matter(8) could carry the inhibitor through into the final product. Such prothrombin or Factor VII would then be "contaminated" and require different conditions for activation than more highly purified preparations or even preparations of low purity but not possessing the postulated inhibitor. Deutsch and Schaden(9) reported that their Factor VII preparation has not been converted to thrombin in 25% sodium citrate solution. The preparation was obtained by adsorbing the active material on barium sulfate and eluting with sodium citrate. These are the conditions under which we obtained our inhibitor. Probably Factor VII may after all be converted to thrombin in 25% sodium citrate when the conditions for its concentration exclude the inhibitor. This is even more likely in view of the fact that prothrombin can be obtained from Factor VII preparations (Ba adsorbed and citrate eluted) with the use of mitochondria (10). If a Factor VII preparation made with the use of barium adsorption can first yield prothrombin and then thrombin there must be some reason why it does not yield thrombin directly in 25% sodium citrate solution. Moreover the methods used in our purification of the prothrombin employed in this work have been applied for obtaining a prothrombin conversion accelerator from serum. This preparation *does* yield thrombin from a non-prothrombin precursor in 25% sodium citrate(11).

The significance of prothrombin activation in 25% sodium citrate solution may not be much appreciated if the view is taken that "the conditions are so unphysiological." However, this activation is the first unequivocal demonstration of the autocatalytic activation of prothrombin. In our work we are considering the possibility that all prothrombin activation is autocatalytic or stated in another way that thrombin is the only enzyme concerned with the activation of prothrombin whether this occurs in sodium citrate or any

other solutions.

Summary. Activation of purified prothrombin in 25% sodium citrate solution can be inhibited by use of a small amount of serum or plasma or barium carbonate eluate. It is postulated that plasma and serum contain an inhibitor(s) that can retard autocatalytic activation of prothrombin.

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Received June 10, 1957. P.S.E.B.M., 1957, v95.

Effect of Ca^{++} and Mg^{++} on Anticomplementary Sera in Complement-Fixation Tests. (23340)

GLADYS M. GNESH (Introduced by Gilbert Dalldorf)

Division of Laboratories and Research, N. Y. State Department of Health, Albany

The observation that mouse liver powder reduces anti-complementary (AC) activity of certain human sera(1) prompted a search for the active principle in the powder. Data have been obtained that indicate that Ca^{++} and Mg^{++} , both of which activate complement (2-8), may be involved.

Materials and methods. Since anticomplementary properties have been attributed to a relative increase in gamma globulin(9-15), 3 lots of commercial gamma globulin were used in the present study. Normal mouse liver powder was prepared and used as described previously(1). The chelating agent for removing Ca^{++} and Mg^{++} from liver powder was an isotonic aqueous solution of ethylene diamine tetra acetate (EDTA), pH 7.8(6). Calcium chloride and magnesium sulfate were used to prepare varying concentrations of Ca^{++} and Mg^{++} . All the reagents were diluted with 0.85% sodium chloride solution(16a). The technic for the complement-fixation test was unchanged(1). AC activity of gamma globulin was measured by its effects on one, two, and three 50% units of complement. According to our standards(1), 85% hemolysis with two 50% units of complement indicates

AC activity. Titrations were also made to determine the effect of Ca^{++} and Mg^{++} on the 50% unit of complement(16b). Preliminary incubation for 24 hours at 3-6°C was followed by 30 minutes at 37°C for hemolysis.

Results. Fifty mg of mouse liver powder were shaken with 1-ml amounts each of distilled water, 0.85% sodium chloride solution, alcohol, ether, and acetone for one hour at room temperature to determine the solubility of the active principle. The mixtures were centrifuged, and the sediment and supernate of each dried in a vacuum desiccator over CaCl_2 . Commercial gamma globulin solution was diluted 1:128 and 1 ml of this dilution, which was known to be AC, was shaken for one hour at room temperature with each of the dried materials, centrifuged, and tested for AC activity. The saline extract was the most effective in removing AC properties from the gamma globulin (Table I).

The active substance in the saline extract was dialyzable through a cellulose membrane.* Passage through an ion exchange

* Visking cellulose sausage casing, purchased from Visking Corp., Chicago, Ill.

TABLE I. Hemolysis Obtained with γ -Globulin Diluted 1:128 and Exposed to Extracts of Mouse Liver Powder.

Solvent	Hemolysis with one, two and three 50% units of complement					
	Dried supernate			Dried extracted liver powder		
	1 u	2 u	3 u	1 u	2 u	3 u
Distilled H ₂ O	15	25	60	15	20	45
.85% saline sol.	60	95	100	10	20	55
95% alcohol	0	10	40	25	35	75
Ether	0	0	15	0	0	10
Acetone	10	75	95	10	15	35
Controls:						
γ -globulin treated with mouse liver powder	25	65	75			
γ -globulin untreated	5	10	30			

Tests were incubated for 24 hr at 3-6°C.

resin (XE64, Na cycle, amberlite 200 mesh) resulted in loss of activity. Heating the dried saline extract to 100°C for 10 minutes slightly enhanced its ability to remove AC properties. The ash of untreated liver powder removed some AC properties.

Several 200-mg portions of mouse liver powder were treated for one hour at room temperature with varying saline dilutions of the EDTA solution, centrifuged, and the liver powder sediment washed 3 times with distilled water. The lowest concentration, 0.002 M solution of EDTA, removed little of the active substance, while the highest, 0.032 M, removed practically all of it.

Thus, the active substance in mouse liver powder appeared to be a divalent cation, possibly Ca⁺⁺ or Mg⁺⁺. Gamma globulin was diluted with saline solution containing vari-

ous concentrations of these ions and the AC activity was removed in a manner similar to the action of mouse liver powder. The ions were used in a ratio of 1 part Ca⁺⁺ to 3 parts Mg⁺⁺. The effect of Ca⁺⁺ alone was much less pronounced than that of Mg⁺⁺; the maximum effect was obtained when the two were combined.

Thirty-five human unfractionated sera were diluted 1:4 with saline solution containing varying concentrations of Ca⁺⁺ and Mg⁺⁺ and tested for AC activity. These ions markedly influenced activity of the sera with complement, both increasing and decreasing hemolytic activity. Optimum amounts for enhancing hemolytic activity varied with individual specimens. A few examples are shown in Table II.

The optimum amounts of Ca⁺⁺ and Mg⁺⁺ for enhancement of the hemolytic activity of complement alone were determined by including varying concentrations in the saline diluent for each reagent in the hemolytic system. These concentrations were incorporated in the saline diluent for all reagents and 28 human sera were tested for AC activity which was increased in 25 of the 28 sera.

Discussion. The experiments implicate Ca⁺⁺ and Mg⁺⁺ among the components of mouse liver powder most active in removing AC properties from serum. Varying the concentrations of these ions in the diluent for individual sera markedly affected their activity with complement, both increasing and decreasing hemolysis in absence or presence of antigen. From these observations, it is evi-

TABLE II. Results with Individual AC Sera Treated with Varying Concentrations of Ca⁺⁺ and Mg⁺⁺.

Serum No.	50% units of complement	% hemolysis obtained with varying concentrations of Ca ⁺⁺ and Mg ⁺⁺												
		*Ca: .03	.023	.02	.017	.013	.01	.007	.00375	.0027	.0017	.0033	None (control)	
		*Mg: .09	.07	.06	.05	.04	.03	.02	.01125	.008	.005	.001		
1	1 unit	20			45			30		20	20	15	10	
2		40	40	45	50	50	50	50	15				0	
3	2	0	10	20	20	45	40	65	70				50	
4		5			5			0		0	5	25	40	
5		90	90	90	95	90	95	85	55				40	
6		85			90			80		70	70	65	45	
7		95			80			55		35	30	5	5	
8		70	45	50	30	30	30	35	25				5	
9	3	50			55			45		15	10	0	0	

* These figures relate to molarity.

The tests were incubated for 24 hr at 3-6°C.

dent that the powder is superior to the ions alone in that it removes AC properties without a disproportionate drop in the antibody titer of the serum(1).

The ions cannot be used in the concentrations which enhance the hemolytic activity of complement alone because the inclusion of these amounts in all of the reagents involved in complement-fixation tests tends to increase AC activity in the majority of the sera. This same difficulty was encountered by Maltaner and Almeida(17). Some investigators determine the complement unit in the presence of Ca^{++} and Mg^{++} and then increase the amount of complement used in the diagnostic test sufficiently to overcome AC activity(18-21). The concentrations for eliminating AC activity would need to be adjusted for each individual specimen which would not be practical. Further studies may disclose that optimum ranges for different degrees of AC activity can be determined. The effect on specific antibody will need to be studied further.

It seems pertinent to pose the question at this time as to what produces AC activity. Do some sera, lacking Ca^{++} and Mg^{++} , remove these ions from the complement and thus inhibit its activity? The data suggest that artificial replacement of the ions either restores or in some way enhances hemolytic activity of complement to overcome AC activity. Experiments have shown that Ca^{++} and Mg^{++} do not have a lytic effect on either sensitized or unsensitized sheep cells in the absence of complement.

Summary. 1) The action of normal mouse liver powder in removing AC properties from sera appears to be associated with Ca^{++} and Mg^{++} . These ions eliminated AC activity from γ globulin in a manner similar to that of mouse liver powder. 2) Varying the amounts of Ca^{++} and Mg^{++} with individual

human sera produced marked changes in their reactivity with complement. The introduction of Ca^{++} and Mg^{++} is less practical at present than the use of liver powder to eliminate AC properties.

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Received March 1, 1957. P.S.E.B.M., 1957, v95.

Attempt to Stimulate Lymphatic Gland Changes of Fowl Typhoid With Adrenal Cortex Extract.* (23341)

HENRY W. GARREN (Introduced by G. Howard Satterfield)

Department of Poultry Science, North Carolina State College, Raleigh

Young chickens infected with fowl typhoid organisms have hyperactive adrenals which are accompanied by an involution of the thymus and bursa of Fabricius(1). The latter lymphatic gland undergoes a peculiar loss of cells from the follicles, a loss that appears to be closely correlated with the degree of cortical activity. Selye(2) presented evidence indicating that the cortical hormones possibly were responsible for lymphatic involution in animals subjected to stress stimuli.

The present study was conducted to determine if exogenous adrenal cortex extract (ACE) would cause in lymphatic glands the weight losses and histological changes observed for these glands from chickens having fowl typhoid.

Methods. At the beginning of each of 2 trials, 5 groups of chickens were selected. In trial A, each of these groups contained 5 individuals and in trial B, 12 individuals. These groups were comparable with respect to age, sex, and weight. The birds of trial A were 39 days old at the start and those of

trial B were 35. The range in initial, average weight for groups of the first trial was 484 to 491 g and 312 to 316 g for the second. In both trials, Group 1 constituted the control while Group 2 was injected intramuscularly with a 1 ml suspension of *Salmonella gallinarum* organisms. Groups 3, 4, and 5 were given ACE daily in single, intramuscular dosages of 0.25, 0.50, and 0.75 ml respectively in trial A. These groups of trial B received respective dosages of 1, 2, and 3 ml daily. Because of the large quantities employed in the latter trial, each of the daily levels was divided into 3 equal doses and given at 8 hour intervals. Insofar as possible all groups were maintained under the same environmental conditions. Whenever ACE was administered to Groups 3, 4, and 5 the controls were given sterile saline injections. The organisms used for infecting Group 2 with fowl typhoid were grown on proteose No. 3 (Difco) slants for 24 hours at 37°C. The colonies on each slant were washed from the agar with sterile saline. This suspension was diluted to 14% light

TABLE I. Effect of Fowl Typhoid and Daily Injections with ACE on Gland and Body Weights of Young Chickens.

	Final wt, g	Pituitary	Adrenal	Bursa	Thymus	Spleen	Liver
		mg/100 g of body wt				g/100 g body wt	
<i>Trial A</i>							
Control	564		10.4	152	560		
Typhoid	377		23.6	67	150		
ACE, 1/4 ml	540		11.9	102	360		
1/2	534		10.4	123	380		
3/4	527		9.6	91	350		
Stand. errors	23.7		1.1	18.7	5.8		
<i>Trial B</i>							
Control	412	1.28	13.6	82	690	.28	2.74
Typhoid	311	1.49	19.1	62	350	.60	5.26
ACE, 1 ml*	353	1.36	12.1	62	420	.28	3.22
2 *	341	1.65	12.7	60	440	.25	3.40
3 *	341	1.57	10.9	60	410	.31	3.75
Stand. errors	13.9	.08	.9	4.4	34	.04	.12

* Divided into 3 equal doses and given at 8 hr intervals.

* Contribution from Dept. of Poultry Science, N. Carolina Agri. Exp. Station as Journal Paper 798.

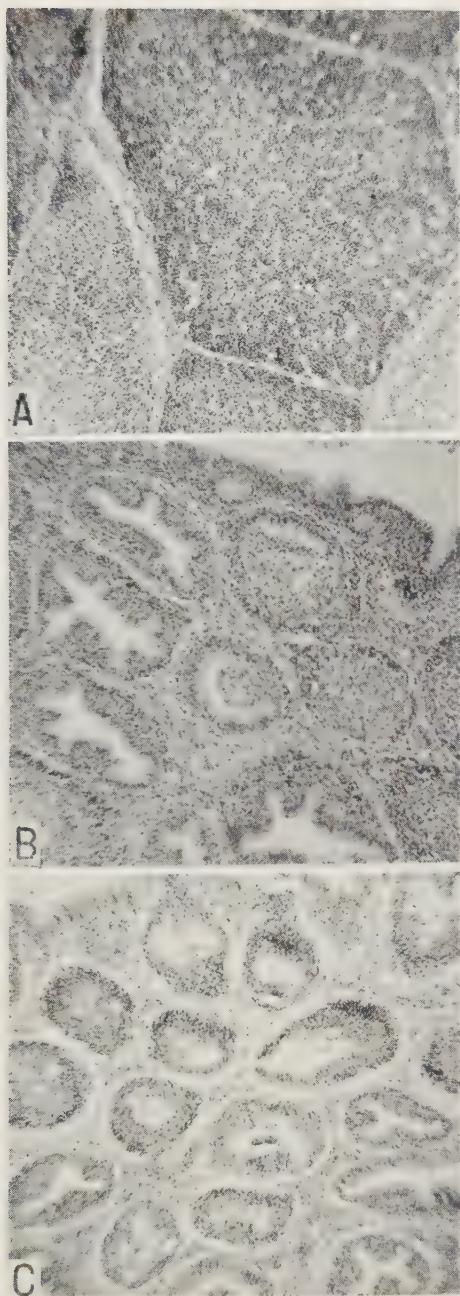


FIG. 1. Photomicrographs of bursa of Fabricius from a control chicken, A; from one infected with fowl typhoid, B; and from one inj. daily for 6 days with 2 ml of adrenal cortex extract, C. ($\times 75$).

transmission on a Coleman spectrophotometer model 14, using a PC-4 filter, at wave length of 650 $m\mu$. Each ml of ACE (Upjohn) contained the biological activity equivalent to

100 μ g of hydrocortisone acetate. When a chicken in Group 2 died, its control was sacrificed in each of the 4 remaining groups. In addition to noting the lymphatic gland changes, the effect of ACE on body weight and on weight and histology of the pituitary, thyroid, adrenal, gonads, liver, spleen, and thymus was also recorded. Statistical analysis of the gland and body weights included an analysis of variance. Standard errors of the mean in the Table were calculated from the average bird-to-bird variance for each set.

Results. As it developed, the *S. gallinarum* culture used in trial A was very virulent; of the infected group, one chick died on the third day following infection, 2 on the fourth, and 2 on the sixth and final day of the trial. In trial B the culture had apparently become less virulent since none of the infected chickens died during the experimental period of 6 days. However, fowl typhoid was diagnosed in these chickens when they were autopsied.

Gland and body weight data obtained in the 2 trials are given in Table I. In trial B, control chickens were smaller and possessed smaller bursae than control chickens of trial A. This differential in body weight is not unusual in chickens of this age grown at 2 different periods of time. A subsequent study has revealed that frequent handling, as present in trial B, can cause a mild but typical stress response as indicated by reduction in body weight, accompanied by an adrenal hypertrophy and a lymphatic involution. It has also been found that the bursa of Fabricius undergoes a rapid loss in weight when chickens are subjected to relatively short periods of stress(3).

Close comparisons between trials A and B are not possible because of differences in bird size and pathogenicity of the infecting organisms. Then too, the multiple injections employed in trial B would be expected to make these larger levels of ACE more effective. However, general comparisons can be made. In both trials the groups infected with fowl typhoid failed to make gains in body weight and the final weights for these groups were significantly less than the control. A weight loss of 109 g occurred for this group

in trial A. For a given trial, the group having fowl typhoid also displayed larger adrenals and smaller bursae than the control. In terms of mg per 100 g of body wt, the pituitary was larger for the group with fowl typhoid in trial B when this gland was considered. Similar weight changes and histological alterations associated therewith have been reported(1).

In both trials, all levels of ACE administration resulted in weights for thymus and bursa of Fabricius which were significantly less than observed for the control. In trial B, 1 ml daily was sufficient to cause a reduction in weight of the bursa comparable with that noted for the bursae of chickens with fowl typhoid, but was not sufficient to cause comparable histological changes. For the latter purpose, 2 ml daily were necessary. Three ml daily caused histological changes in the bursa which were slightly more severe than generally observed in this gland from chickens of the fowl typhoid-infected group. None of the levels of ACE employed was sufficient to cause a decrease in thymus weight comparable with that observed for groups having fowl typhoid. Actually for a given trial, the largest level of ACE administered failed to cause a significantly greater decrease in thymus weight than did the smallest level administered. The reason for this relationship is not known, particularly in view of the marked involution of the thymus from individuals having fowl typhoid. Possibly the thymus tissue of healthy chickens has a greater regenerative capacity than the same tissue of infected chickens. Then too, other physiological changes besides increased corticoid production may occur with fowl typhoid infection and contribute to the loss of cells from the thymus. And, of course, the highest level of ACE employed in the present study could be lower than necessary to cause a comparable loss of cells from the thymus.

Other results obtained, included (a) ACE injections which caused a significant increase in relative weights of the pituitary gland. This gland was larger than the control on an absolute basis for groups given 2 and 3 ml daily, but not significantly so. No discernible

histological changes were noted. (b) Adrenals of the group given 3 ml of ACE daily were significantly smaller than the control. Less cortical tissue was present in groups given 1, 2, and 3 ml of ACE daily than observed in the control group. (c) The spleen weight and gross histology were unaffected by ACE administration. (d) ACE caused an increase in liver weight. Statistical analysis of the data indicated that weight increased in a linear fashion with larger doses of ACE. The yellow appearance of livers from groups injected with ACE suggested that increase in liver weight may have resulted from an increased fat deposition. However, no analysis of fat content was made. Enlarged livers and spleens of groups having fowl typhoid constitute characteristic lesions of the disease (4). (e) ACE administration at all levels caused a retardation in body growth. This reduction was significant for groups given daily levels of ACE in amounts of 1, 2, and 3 ml. This observation suggests the interesting possibility that the high levels of corticoids apparently occurring naturally in chickens with fowl typhoid may contribute to the marked body weight losses associated with the disease. Restriction of feed and water intake to that consumed by chickens with fowl typhoid is not sufficient to cause comparable weight losses noted for chickens with the disease(1). (f) The levels of ACE administered had no significant effect on the weight of the comb, gonads, or thyroid.

Summary. Daily levels of ACE for 6 days in amounts of 0.25 to 3 ml caused a significant reduction in weight of the thymus and the bursa of Fabricius. Two ml daily were adequate to cause both the weight loss and histological changes occurring in the bursa of Fabricius from chickens with fowl typhoid. Other significant changes observed for the groups given 1, 2, and 3 ml daily included: decrease in body weight, increase in relative pituitary weight, reduction in adrenal weight (less cortical tissue), and increase in liver weight which bore a linear relationship with the level of ACE administration.

The author wishes to thank Dr. Kenneth B.

Hass of the Upjohn Co. for supplying the adrenal cortex extract, Dr. Henry Lucas for the statistical analysis, Miss Marion Smith for technical assistance, and Mr. John Mattox for the photomicrographs.

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Received March 20, 1957. P.S.E.B.M., 1957, v95.

Evidence for Adrenergic and Cholinergic Components in Milk Let-Down Reflex in Lactating Rat.* (23342)

CLARK E. GROSVENOR† AND CHARLES W. TURNER

Department of Dairy Husbandry, University of Missouri, Columbia

A cholinergic component apparently is involved in activation of the hypothalamo-hypophyseal mechanism for release of antidiuretic and luteinizing hormones and, in addition, an adrenergic link has been proposed for luteinizing hormone and ACTH release(1). However, very little appears to be known about the release of oxytocin from the posterior pituitary gland other than acetylcholine, when injected into the dog, results in increased uterine activity apparently due to liberation of oxytocin(2). Since oxytocin is released as a result of nursing or milking stimuli in lactating animals thereby resulting in milk let-down(3), it seemed of interest to investigate the possibility of cholinergic and adrenergic components in this phenomenon. In the present study, the effects of various blocking agents on let-down in lactating rats have been studied and results compared with normal values previously obtained(4).

Materials and methods. Adult primiparous lactating rats weighing 200-300 g were assayed for milk let-down according to the method of Grosvenor and Turner(4). These were divided into 3 groups: *Ergot Group*. Each mother received a single subcutaneous injection of one of the following ergot drugs‡

10 minutes prior to nursing: methylergonovine tartrate (Methergine), 1-2 mg/kg; an equi-proportional mixture of methanesulfonate salts of dihydroergocornine, dihydroergokryptine and dihydroergocristine (Hydergine), 2-4 mg/kg; dihydroergotamine methanesulfonate (DHE 45) 2-4 mg/kg. In addition, some mothers received .2 USP/kg oxytocin ("P.O.P." Armour) subcutaneously 8 minutes after Methergine or 10 minutes after Hydergine and DHE 45, i.e., immediately before nursing. *Dibenamine Group*. Each mother received a single subcutaneous injection of Dibenamine HCl§ which was administered to one group in dose of 40-60 mg/kg 15-60 minutes prior to nursing and 60-80 mg/kg 120 minutes prior to nursing to a second group. A third group was injected with 80 mg/kg 120 minutes before nursing followed by .2 USP/kg oxytocin subcutaneously immediately before the animals commenced to nurse. *Atropine Group*. Each animal in this group received a single subcutaneous injection of atropine sulfate in dose of 700 mg/kg either 15-60 minutes or 150-220 minutes prior to nursing. Eighteen of these were anaesthetized with Nembutal (45 mg/kg) and oxytocin administered intravenously while the young were actively sucking. Gross observations were made on effects of each drug on behavior of the animals.

Results. Ergot Group. All ergot drugs, in dosage employed in the present study, sig-

*Contribution from Department of Dairy Husbandry, Mo. Agr. Exp. Station. Journal Series No. 1743. Approved by Director.

† Postdoctoral Fellow of N.I.H. This investigation was supported in part by research grant from the Public Health Service.

‡ Kindly supplied by Sandoz Chemicals, N. Y.

§ Kindly supplied by Smith, Kline and French Laboratories, Philadelphia.

TABLE I. Milk Removal by Nursling Rats on 14th Day Postpartum.

Treatment	No. of rats	Time of treatment prior to nursing (min.)	Avg wt of litters (g)	Avg wt of milk (g)	Avg %	
					Milk wt Litter wt	SE
Control†	40		144.0	5.5	3.8	.19
Methergine, 1-2 mg/kg	10	10	132.8	1.7	1.3	.31*
<i>Idem</i> , then Oxytocin, .2 USP/kg	5	10	162.8	6.8	4.2	.19
Hydergine, 2-4 mg/kg	13	10	150.0	2.6	1.6	.35*
<i>Idem</i> , then Oxytocin, .2 USP/kg	5	10	177.6	8.7	4.9	.28
DHE 45, 2-4 mg/kg	10	10	151.0	3.3	2.2	.38*
<i>Idem</i> , then Oxytocin, .2 USP/kg	5	10	155.2	7.9	5.1	.46
Dibenamine, 40-60 mg/kg	11	15-60	187.0	7.6	4.1	.39
" , 60-80 "	10	120	174.5	4.2	2.3	.29*
<i>Idem</i> , then Oxytocin, .2 USP/kg	5	120	177.6	9.0	5.1	.37
Atropine, 700 mg/kg	8	15- 60	165.1	5.0	3.4	.47
<i>Idem</i>	10	150-220	159.0	1.2	.8	.11*
Atropine, 700 mg/kg, then Oxytocin, .05 USP/kg i.v. into Nembutal anaesthetized mothers	9	150-220	166.6	6.1	3.7	.40
Atropine, 700 mg/kg, then Oxytocin, .1 USP/kg i.v. into Nembutal anaesthetized mothers	9	150-220	173.9	6.1	3.6	.23

* P < .01.

† Data from Grosvenor and Turner (4).

nificantly inhibited milk let-down in lactating rats when compared with control values previously obtained (4). Methergine was the most potent inhibitor, followed by Hydergine and DHE 45, in that order (Table I). All treated animals showed normal behavioral patterns in gathering their young preparatory to nurse and during the nursing period and only those treated with Methergine showed any visible sign of possible central nervous system depression evidenced primarily by lethargy. No animal under any treatment showed signs of central excitation.

Dibenamine Group. It was noted all rats of this group reacted to treatment with Dibenamine by being highly restless and irritable for approximately 30-45 minutes post-injection after which they gradually became calm and assumed normal behavior. Such excitation did not, however, affect nursing behavior or affect let-down response in those animals permitted to nurse during the period of maximum excitation (Table I). Milk let-

down was significantly inhibited when 60-80 mg/kg Dibenamine was administered 120 minutes prior to nursing. There was no apparent difference in inhibitory effects of the 2 doses.

Atropine Group. The response of lactating rats to atropine was one of extreme excitability commencing 30-60 minutes post-injection and lasting for several hours. Milk let-down was significantly inhibited in those rats injected 150-220 minutes before nursing but was not inhibited in those treated 15-60 minutes before nursing.

Inhibition following ergot or Dibenamine administration was reversed when .2 USP/kg oxytocin was injected subcutaneously just prior to nursing resulting in normal or above normal yields of milk. In atropinized anaesthetized animals normal yields also were obtained when .05 or .1 USP oxytocin was injected intravenously.

Discussion. Neural pathways involved in release of oxytocin from posterior pituitary

gland following nursing stimuli are obscure though the brain, hypothalamus and supra-opticohypophyseal tract apparently are involved(1). It has been shown that adrenalin prevents release of antidiuretic hormone(5,6) and may perhaps inhibit milk let-down by preventing discharge of oxytocic hormone(7). However, in the present study sympatholytic agents, like some ergot alkaloids and Dibenamine, inhibited milk let-down indicating the possibility of an adrenergic component involved in this reflex. That the inhibition is not peripheral is evidenced by observations that exogenously administered oxytocin was capable of eliciting normal milk yields when injected after any sympatholytic tested. However, all ergot drugs have complex central nervous system effects at various dose levels which are not well understood so the possibility exists ergot alkaloids may inhibit milk let-down in the rat primarily by central depression such as is obtained with Nembutal(8). Inhibition obtained with Methergine, which has no demonstrable sympatholytic activity(9) supports this concept, for it was noted Methergine-treated animals which showed symptoms of possible central depression were inhibited more in their let-down response than were those treated with either Hydergine or DHE 45. Similar symptoms were noted previously(10) with ergotamine-treated lactating rats. On the other hand, neither Hydergine nor DHE 45 showed visible signs of possible central depression of excitation, yet milk let-down was significantly inhibited. Dibenamine has a direct excitant action on the central nervous system which develops soon after administration but which appears to be independent of its adrenergic blocking action(11). This was apparent in the present study for Dibenamine elicited visible symptoms of central excitation which persisted for 30-45 minutes though milk let-down was not inhibited in any animal who nursed during this period. Let-down, however, was significantly inhibited when the drug was injected 2 hours prior to nursing after which time adrenergic blockade is maximum, or very nearly so(11). It is interesting to note high concentrations of sympathin are present in hypothalamus(12) and therefore possibly the

locus of adrenergic blockade of milk let-down in lactating rats.

Analysis of data obtained from atropine-treated lactating rats points to the possible presence of a cholinergic component in the let-down reflex. However, it might be argued that inhibition of let-down in atropinized animals might be the result of unopposed adrenergic impulses which might evoke such intense constriction of mammary gland blood vessels that circulating oxytocin would be prohibited from reaching the effector myoepithelial cells. It has been shown adrenalin in appropriate doses can block milk let-down response to both endogenous and injected oxytocin(7,13-15). We feel some vasoconstriction of the blood supply of the mammary gland does occur since .1 USP/kg oxytocin injected intravenously failed to elicit greater milk removal than .05 USP/kg for in a previous study the former dose resulted in a 55% greater milk yield(8). However, the observation that .05 USP/kg oxytocin resulted in a normal let-down response would seem to indicate that vasoconstriction is not severe enough to prevent normal let-down, and it is therefore postulated the principal site of blockade is primarily central rather than peripheral. It was apparent that 1-2 hours must elapse from time of injection until cholinergic paralysis of milk let-down is maximum since atropine, when administered 15-60 minutes before nursing, failed to block the response. It is postulated from the results of this study that adrenergic and cholinergic nerve links are present in the neurohumoral arc responsible for discharge of oxytocin from the posterior pituitary gland.

Summary. The amount of milk obtained by litters of lactating rats treated with various ergot alkaloids, Dibenamine or atropine, was compared with control values previously obtained. All ergot drugs tested significantly blocked the let-down response. Methylergonovine (Methergine) was the most potent inhibitor, followed by the dihydroergotoxine complex (Hydergine) and dihydroergotamine (DHE 45), in that order. Results indicate action on central nervous system is probably responsible for some, or perhaps all, milk let-down inhibitory action of ergot drugs. This

observation was based primarily on results with Methergine, a non-sympatholytic compound. Dibenamine evoked symptoms of central excitation which persisted for 30-45 minutes after injection though milk let-down was not inhibited in those animals who nursed during this period. Milk let-down, however, was significantly inhibited when the drug was injected 2 hours prior to nursing, after which time adrenergic blockade has occurred. Atropine induced a highly significant blockade of the let-down response when injected 150-220 minutes before nursing, but failed to block the response when administered 15-60 minutes before nursing. Oxytocin administered after any of these drugs restored normal milk let-down, indicating blockade was central rather than peripheral. Since Dibenamine, Hydergine and DHE 45 are powerful adrenergic blocking agents and atropine a cholinergic blocking agent, it is postulated both adrenergic and cholinergic links are present in the neurohumoral arc responsible for discharge of oxytocin from the posterior pituitary gland.

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Received May 3, 1957. P.S.E.B.M., 1957, v95.

Digestive Action of Gastric Juice Following Various Types of Gastric Resection for Duodenal Ulcer.* (23343)

JOHN F. PERRY, JR., PETER A. SALMON, KAMIL IMAMOGLU, E. G. YONEHIRO, AND OWEN H. WANGENSTEEN

Department of Surgery, University of Minnesota Medical School, Minneapolis.

The present report evaluates operations designed for treatment of duodenal ulcer by comparison of the digestive action of the gastric juice following such procedures. For this purpose the erosive effect of gastric juice on the cat's esophagus has been measured and pepsin and acidity have been determined. These data have been compared with similar data from a study of control non-ulcer patients and another group of patients with un-

treated duodenal ulcer. Previous experiments (1) have shown gastric juice from 72% of untreated duodenal ulcer patients produced grade 3 to 5 digestion of the cat's esophagus during a 2 hour perfusion period. Only 9% of gastric specimens from control non-ulcer patients produced such severe degrees of esophageal erosion. Acidity and pepsin concentrations in the gastric juice of the majority of duodenal ulcer patients were found to be greater than in controls(1). A correlation of the data reported below, together with the observed incidence of ulcer recurrence following the several operative procedures studied

* These researches received support from the U.S.P.H.S., Malignant Disease Research Fund, Arthur and Stella Sanford Fund, and Jay and Rose Phillips Fund.

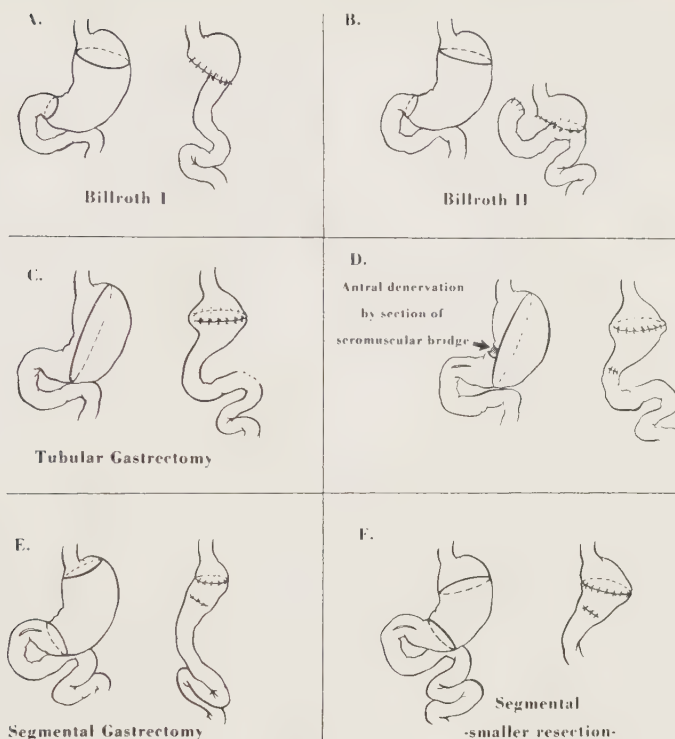


FIG. 1. Types of gastrectomy for duodenal ulcer.

suggests this method may prove to be one of the more precise and critical methods of evaluating the potential protective effect of an operation for duodenal ulcer.

Method. Patients who had undergone various types of gastric resection for duodenal ulcer were readmitted to hospital for overnight aspiration of the gastric contents. All operations had been done 3 months to several years previously. Secretions were collected by continuous suction from the gastric pouch with an inlying gastric tube from 11:00 p.m. to 7:00 a.m. The specimen bottle was kept on ice during the collection period, the volume was noted and the aspirate was then either frozen or subjected to analysis immediately. The pH was determined with a glass electrode and free HCl was determined by titration with 0.1 N NaOH. Pepsin was determined by the hemoglobin substrate method of Anson(2) and expressed as tyrosine activity units per cc of gastric juice. The gastric juice was adjusted to pH 1.6 to 1.7 with dilute HCl. The resultant solution was utilized to perfuse the esophagus of an anesthetized cat

as previously described(1). Degree of digestion of the esophagus at the end of a 2 hour perfusion period was graded after gross and microscopic examination. Grades 0, 1 and 2 represent slight to moderate erosion of the esophagus. Grades 3 and 4 indicate severe degrees of digestion; grade 5 represents perforation of the perfused esophagus.

Types of operative procedures studied are shown in Fig. 1. In both Billroth operations (A & B) a 75% excision of stomach including antrum had been done. Tubular gastrectomy (C)(3) entailed excision of the body and fundus with preservation of the cardia, lesser curvature and antrum. Reconstitution was by transverse gastroplasty. Heineke-Mikulicz pyloroplasty was done only if cicatricial stenosis of pylorus was present. Tubular gastrectomy with antral denervation(D) differed in that vagotomy at the level of the antrum was carried out by section of the narrow seromuscular bridge at the lesser curvature; pyloroplasty was then done routinely. The larger segmental gastrectomy (E)(4) entailed 85 to 90% excision of the acid-pepsin

TABLE I. Eight Hour Night Secretion Following Gastrectomy for Duodenal Ulcer.

TABLE I. Eight Hour Night Section Following Gastric Surgery													
Patients studied				Analysis of gastric aspirate									
Type of gastrectomy	No. of patients	Age		Sex	Vol (ml)		Free HCl (total mEq)		pH		Pepsin (units/ml)		
		Mean	Range		♂	♀	Mean	Range	Mean	Range	Mean	Range	
A. Billroth I	10	53	29-68	5	5	169	40- 510	1.0	0- 10.0	5.2	1.6-7.2	.6	1-2.0
B. " II	12	54	26-69	11	1	163	60- 321	0	0	5.7	3.5-7.4	.5	1-1.8
C. Tubular	20	52	27-73	11	9	360	80- 680	4.6	0- 37.4	3.9	1.6-6.0	.5	1-1.4
D. Tubular with antral denervation	6	62	31-77	4	2	292	80- 390	1.75	0- 10.5	4.3	2.1-7.0	.3	1- .6
E. Segmental (large)	11	52	30-68	9	2	354	45- 670	.2	0- 2.2	5.9	2.5-7.4	.2	1- .6
F. " (small)	21	50	24-76	17	4	316	10-1000	2.36	0- 35.0	4.7	1.2-7.0	.3	1- .8
G. Control (non-ulcer)	81	57	24-78	41	40	256	10- 800	3.04	0- 21.23	3.7	1.4-7.4	.9	1-5.0
H. Duodenal ulcer (untreated)	29	54	27-72	19	10	479	48-1100	19.04	0-110.40	2.1	1.2-4.2	1.7	2-4.1

secreting area of the stomach, with preservation of antrum. Reconstitution was by gastrogastrostomy, and a pyloroplasty was added regularly to facilitate gastric emptying. The smaller segmental gastrectomy (F) differs from (E) only in that the extent of excision of the acid-pepsin secreting stomach was 40 to 60%.

Results. Eighty patients have been studied following various types of gastric resection for duodenal ulcer. The distribution of patients as to type of gastrectomy, age and sex, and the results of analysis of the overnight gastric secretion data are shown in Table I. Table II gives the results of perfusion of the cat esophagus with gastric juice obtained after the various types of gastric resection. Included are data from analysis of gastric secretion of control non-ulcer and untreated duodenal ulcer patients.

A. Following the Billroth I operation, one of 10 patients had demonstrable free hydrochloric acid in the gastric aspirate. This patient and an additional one had pepsin concentrations well within the range observed in patients with active duodenal ulcer. Two of 10 gastric juice specimens on perfusion produced severe grades of erosion of the cat's esophagus. These results indicate that uniformly lower levels of acid peptic digestion have not been achieved by the Billroth I gastrectomy.

B. After the Billroth II gastrectomy, no free HCl was demonstrated in the gastric juice of 12 patients and the pH values were no lower than 3.5 in any instance. Yet 3 patients still had pepsin concentration in the ulcer range. Perfusion of the cat's esophagus with 2 of these specimens caused severe digestion (grades 3 and 5). Control of the acid component has been achieved by this operation but these digestion studies suggest that the gastric juice of some of these patients still possesses digestive activity of the character observed in patients with duodenal ulcer.

C. Free HCl was present in the gastric juice of 6 of 20 patients studied after tubular gastrectomy. In 3 of these the acid values were high and the pH was correspondingly low. Measurements of pepsin revealed values

TABLE II. Digestion of Cat Esophagus with Gastric Juice Obtained after Various Types of Gastric Resection for Duodenal Ulcer. Perfusion at pH 1.6-1.7.

Type of gastrectomy	Total No. of patients	Grade of digestion					
		With No. of patients whose gastric juice activity falls in each category					
		0	1	2	3	4	5
A. Billroth I	10	1	4	3	—	1	1
B. " II	11	4	4	1	1	—	1
C. Tubular	19	4	9	3	1	—	2
D. Tubular with antral denervation	6	2	3	—	1	—	—
E. Segmental (large)	11	1	8	2	—	—	—
F. " (small)	20	2	16	—	1	—	1
G. Control (non-ulcer)	56	15	30	6	2	2	1
H. Duodenal ulcer (untreated)	26	2	4	1	7	6	6

in the range of observations upon patients with active duodenal ulcer in only one instance; yet on perfusion, severe digestion of the cat esophagus followed in 3 instances. In 2 of these, gastric juice was obtained from patients known to have clinical evidence of recurrent ulcer. Persistent free hydrochloric acid in 30% of patients studied, and persistent high digestive activity of the gastric juice in 15% suggest that tubular gastric resection is not a suitable operation for duodenal ulcer.

D. Antral denervation added to tubular gastrectomy failed to provide uniform reduction in acid peptic digestive activity. One of 6 patients had a significant quantity of free HCl but perfusion of the cat's esophagus produced no erosion (grade 0). Digestive activity from the gastric juice of another patient in the series produced a grade 3 esophageal erosion. This group of patients is small; yet, the results suggest that an abnormal digestive potential was present in the gastric juice in at least one of them.

E. After the large segmental gastrectomy (85-90% excision of the acid-pepsin secreting area), the potential for ulceration by proteolytic digestion appears to be well controlled. In only one of 11 specimens studied was free HCl present and the value was small (2.2 mEq.). Pepsin values were low in all instances. Perfusion of the cat esophagus did not result in severe degrees of erosion in any instance.

F. With more limited excision of the acid-pepsin secreting area (40-60%), control of acid peptic digestion has not been as striking as following the larger segmental gastrectomy.

In 7 of 21 specimens, free HCl was present; in one of these because of the large volume (1,000 cc), the mEq. of free HCl was high, totalling 35. Pepsin concentration was not abnormally high in any instance, yet perfusion of the cat esophagus in 2 instances caused severe erosion (grades 3 and 5).

Discussion. The results of an assessment of the potential digestive power of gastric juice correlates well with our clinical experience with these operations in the surgical management of duodenal ulcer. The demonstration of significant acid peptic digestion by gastric juice of some patients studied after Billroth I and tubular operations is compatible with the known incidence of recurrent ulcer following these procedures(5,6). Although high levels of peptic digestive activity can be demonstrated in gastric juice after Billroth II resections, the pH of the juice is usually high due to continued mixing with the alkaline juices coming from the proximal duodenal loop. Thus pepsin activation is not great inasmuch as the pH is not optimal. In the Billroth II operation, therefore, one might reasonably anticipate fair protection against recurrent ulcer, largely through control of the pH. Extensive clinical experience with this operation indicates that the ulcer recurrence is in the area of 2 to 4%. In the large segmental gastrectomy (85-90% excision of acid-pepsin secreting area) uniform control of the acid peptic factor has been achieved in all patients studied. Clinical experience with this operation is comparable(4,7). Over one hundred patients have been followed 4 to 8 years following this operation without a single

recurrent ulcer. Patients having the smaller segmental gastrectomy (40 to 60%) have not been followed for a sufficiently long time to permit full clinical evaluation of the operation(7). No recurrent ulcer has yet been observed; however, significant acid peptic digestion in the gastric juice was observed in 2 of 20 patients (grades 3 and 5; 10%).

It is too early, therefore, to conclude that the smaller segmental gastric resection (40 to 60%) will protect against recurrent ulcer in the same measure as the larger segmental resection.

Summary and conclusions. Measurements of acid and pepsin values and determination of degree of digestion attending perfusions of the cat esophagus with gastric juice, obtained from patients after gastric resection for duodenal ulcer, have been made. These results indicate that esophageal perfusion employing the gastric juice of patients previously operated upon is a sensitive method for evaluating the likely protective effect of such operations against recurrent ulcer. Following procedures known to be associated with recurrent ulcers, gastric juice from some patients can be shown to have proteolytic digestive power not unlike that seen with untreated duodenal ulcer. After operations known from clinical experience to protect fully against recurrent ulceration,

the gastric juice of patients uniformly shows acid peptic digestion in the range of normals. This technic of assessment indicates that the protection afforded by some gastric resections is inadequate and that recurrence is likely to follow. The operation affording the greatest protection is definitely the larger (85-90%) segmental resection. Lesser grades of protection appear to be provided by the smaller (40-60%) segmental resection and the Billroth II procedure. Next in line would appear to be the protection afforded by tubular resection with antral denervation. Least protection against recurrent ulcer would appear to be afforded by tubular gastric resection and the Billroth I operation.

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Received May 16, 1957. P.S.E.B.M., 1957, v95.

Convulsions and Bizarre Behavior in Monkeys Receiving Chlorpromazine. (23344)

CARL F. ESSIG AND WOODROW W. CARTER (Introduced by A. Wikler)
NIMH Addiction Research Center, U.S.P.H.S. Hospital, Lexington, Ky.

That chlorpromazine may have convulsant actions in addition to its "tranquilizing" properties is suggested by a number of reports in the literature. Thus, in patients with epilepsy, Bonafede(1) reported that although addition of chlorpromazine to the therapeutic regimen without change in anticonvulsant medication did not augment seizure rates, reduction of the dosage of anticonvulsant drugs while chlorpromazine was being given was followed by an increased number of seizures in 26 out of 78 patients, one of whom died in status

epilepticus. Bente and Itil(2) observed that intravenous injection of 50 mg of chlorpromazine augmented paroxysmal activity in the electroencephalograms of epileptic patients, and in one of their subjects a major seizure occurred later. Likewise, Kopeloff *et al.*(3) found that, in monkeys with focal cortical cicatrices produced by topically applied discs of alumina cream, intravenous injection of 2 or 10 mg/kg of chlorpromazine accentuated focal spike and sharp wave electroencephalographic activity, and on some occasions also

induced "spontaneous" seizures. Also, Das *et al.*(4) noted that in cats with midbrain transections ("cerveau isolé" preparations), intravenous injection of 0.2-0.5 mg/kg of chlorpromazine produced transient high voltage spike-wave discharges, and in 2 of these with basis pedunculi intact, convulsions developed. In addition, attention may be directed to the occasional occurrence of psychoses in man during therapy with chlorpromazine, as reported by Mality *et al.*(5).

These data suggest that chlorpromazine does exert convulsive actions, and that this property constitutes a hazard in therapy only in patients with preexisting epilepsy. However, in the course of a study designed for a different purpose (an attempt to produce a Parkinsonian syndrome) we found unexpectedly that, at certain dose levels, chlorpromazine can produce both seizures and hallucinatory-like behavior in "normal" monkeys. Since these findings may be of clinical significance they are reported briefly herewith.

Method. Four monkeys (*Macaca mulatta*) weighing 7.3 to 13.4 kg were used. The first dose of chlorpromazine (25.0 mg/kg) was given intramuscularly. Thereafter, the drug was given orally once-a-day. A uniform dose schedule was not practicable; in general, the

dose was increased from 25 to 77 mg/kg for 13 to 22 days (Fig. 1). The temporal incidence of major convulsions was recorded in a special activity cage in which a spring-mounted floor was attached to a phonograph crystal pickup. The amplified crystal output drove an EEG oscillograph and pen-writing unit which recorded on very slowly moving paper (0.5 mm/sec). All animals except monkey No. 1 were monitored in this cage for 6 to 11 days before the drug was administered. Activity recording was continuous during the period of drug administration and withdrawal. Repeated attempts to induce seizures by vigorously prodding the animals were made throughout the experiment. Monkey No. 1 was not placed in the activity cage until the dose had been increased to 77 mg/kg and the first unexpected seizure was actually witnessed. Electroencephalograms were obtained occasionally while the animals were receiving the larger doses of chlorpromazine.

Results. Gross behavioral changes observed after administering the drug warrant brief summary. Grimacing and unprovoked aggressiveness, characteristic of the Rhesus monkey, were characterized. However, if animals were handled they resisted and tried to

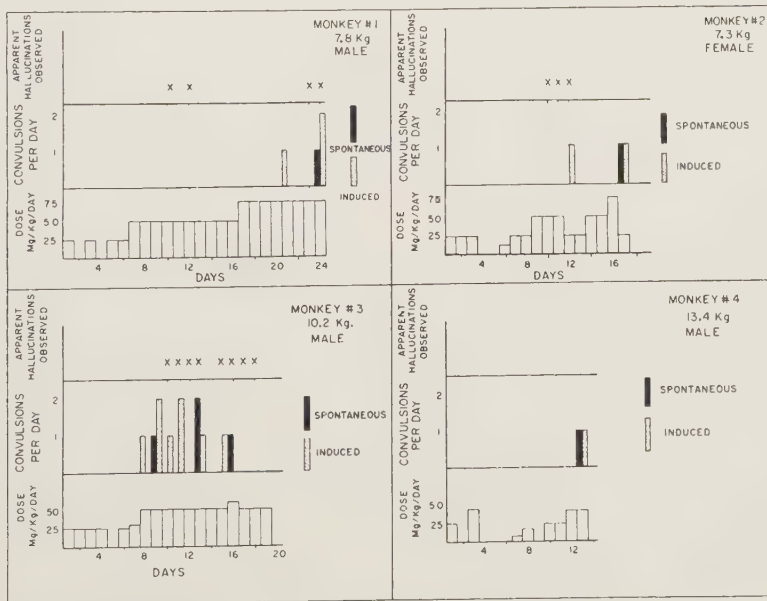


FIG. 1. Dose regimen and seizure incidence.



FIG. 2. Spontaneous convulsion, monkey No. 4.

bite. Tremulousness during movement or effort was a striking and consistent motor effect observed after the drug. This "action" tremor was rapid in frequency, small in amplitude, and involved all extremities.

Neither spontaneous nor induced seizures (observed after prodding or chasing) occurred before the drug was administered. In contrast, both of these kinds of major convulsions were observed in every animal while taking chlorpromazine. The total number of convulsions per animal ranged from 2 to 12. Details of seizure occurrence and dose regimen are depicted in Fig. 1. None of the animals had convulsions at the 25 mg/kg dose level. On the other hand, convulsions occurred in every animal on higher dosages which varied from 44 to 77 mg/kg a day. Animal No. 1 may have had convulsions when given 52 mg/kg of chlorpromazine, but activity data is lacking as indicated under method. The seizures involved all 4 extremities in rapid myoclonic jerking which lasted 30 to 80 seconds and probably resembled metrazole more than strychnine induced convulsions. The pattern as recorded in the activity cage is shown in Fig. 2. The longest interval between last dose of drug and final induced convulsion was 12 hours (monkey No. 1). In addition, animal No. 3 stopped having convulsions 3 days before the final dose of chlorpromazine. Seizures, therefore, were interpreted as not being due to withdrawal of chlorpromazine. Furthermore, the

animals were not rendered permanently epileptic.

During these experiments the animals lost weight. However, daily administration of a multivitamin preparation (2 Unicaps) plus pyridoxine (20 mg) did not prevent seizures in the only animal treated in this way.

Monopolar scalp lead electroencephalograms did not reveal substantial differences from control tracings when the animals were quiet. Unfortunately, EEGs recorded during the occurrence of myoclonic jerks or focal seizures were obscured by muscle artefacts.

Bizarre behavior suggestive of hallucinations was observed in 3 monkeys. Monkey No. 3 searched the cage floor as though looking for an imaginary object, and occasionally leaped backward as if he were afraid of something. At other times, between seizures, the animal would repeatedly reach out and apparently grasp an imaginary object which would often be carried to his mouth. In animals Nos. 1 and 2, prominent movements of head and eyes resembling oculogyric crises and retrocollic spasms were observed, but these episodes were difficult to differentiate from the hallucinatory-like behavior already described.

The chief behavioral changes compatible with a Parkinson-like syndrome were hypokinesia as well as increased flexion posture of trunk and limbs. Facial masking was equivocal unless a reduction of grimacing is inter-

preted in this manner. Repeated examination of the animals failed to reveal cogwheel rigidity or a tremor at rest.

Discussion. The fact that convulsions did not occur at the 25-mg/kg dose level indicates roughly that the greater the dose of chlorpromazine, the greater is the likelihood of seizures. Although the doses used in our experiments are quite high and exceed the usual dosages ordinarily employed in clinical practice they are comparable to the human dosage regimen (3400 mg of chlorpromazine in one day) reported by one clinic(6). Species difference prohibits quantitative extrapolation of these data to the clinic. However, the current widespread use of chlorpromazine warrants speculation as regards at least 2 clinical situations, (1) in epilepsy, and (2) in management of barbiturate and alcohol withdrawal(1,7,8,9). The use of chlorpromazine or any compound with convulsant properties may be hazardous in these situations.

Summary. Chronic administration of chlorpromazine in doses ranging between 44 and

77 mg/kg caused major convulsions in 4 non-epileptic monkeys and induced behavior suggestive of hallucinations in 3 of the same animals. The possible clinical significance of these findings was discussed.

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Received May 27, 1957. P.S.E.B.M., 1957, v95.

A Method for Evaluating both Non-Narcotic and Narcotic Analgesics. (23345)

ESTELLE SIEGMUND, RICHARD CADMUS AND GO LU
Department of Pharmacology, Ethicon, Inc., Somerville, N. J.

A simple and reliable method for evaluating non-narcotic analgesics in laboratory animals has long been desired. We, therefore, have developed the following method for its testing. The method is based upon the antagonism by both non-narcotic and narcotic analgesics of a "syndrome" induced by intraperitoneal injection of a phenyl quinone. The use of a similar syndrome produced by organic iodinated compounds has been reported by Vander Wende and Margolin(1) for testing narcotic analgesics in rats. However, the method was claimed not to be suitable for evaluating non-narcotic analgesics.

Method. In CF #1 male mice, a typical "syndrome" is produced by intraperitoneal injection of 0.25 ml of 0.02% aqueous solu-

tion of 2-phenyl-1,4-benzoquinone, henceforth referred to as phenyl quinone. This compound is dissolved in 5% ethyl alcohol and distilled water, and the solution maintained at 37°C. The "syndrome" is characterized by intermittent contractions of the abdomen, twisting and turning of trunk and extension of hind legs, beginning 3 to 10 minutes after injection and persisting for more than one hour. Only those mice that exhibit the "syndrome" repeatedly within 10 minutes following injection of phenyl quinone are used. All untreated mice will exhibit the "syndrome" at least once in a 5 minute period. Therefore, after administration of the test drug, a 5 minute observation period is repeated at 15-minute intervals. As the "syn-

TABLE I. Antagonism to the "Syndrome" of Non-narcotic Analgesics in Mice.

Drug*	Form administered	ED ₅₀ (C.L., p = .05)† mg/kg	Time of peak effect (min.)
Sodium acetylsalicylate	Aqueous solution	182 (137-242) 160 (130-197)	30 45
Acetylsalicylic acid	Gum tragacanth suspension	165 (133-204)	30
Acetophenetidin	<i>Idem</i>	260 (186-364)	30
Aminopyrine	Aqueous solution	98 (80-120)	15

* Drugs are admin. orally.

† C.L. = Confidence limits.

drome" occurs, the mice are removed from the cage. Those remaining are considered to show an analgesic effect. Several non-toxic graded doses of the substance being tested are spaced logarithmically and administered to different groups of mice. The dose-response curve is obtained by basing observations on the all-or-none response. The ED₅₀'s are determined according to the Litchfield and Wilcoxon Method(2).

Results. By this method, the approximate time of peak effect of the drug being tested can also be estimated. The ED₅₀'s and the time of peak effects for the 3 non-narcotic analgesics are summarized in Table I.

The method is also suitable for testing narcotic analgesics and the data obtained are compiled in Table II.

It was found that the "syndrome" can also be prevented if the analgesics are administered before injecting the phenyl quinone. No attempt was made to explore possible reuse of the mice.

In preliminary studies, attempts were made to elucidate the mechanism of this "syndrome" and its possible relation to pain sensation. It was found that barbiturates (phenobarbital sodium, pentobarbital sodium or butobarbital sodium), at doses producing loss of body righting reflex, did not prevent the "syndrome." Tranquilizers (meprobamate or

chlorpromazine hydrochloride) prevented the "syndrome" only at doses producing marked depression, as did ethyl alcohol. Mephenesin prevented the "syndrome" only at paralyzing doses. However, since both non-narcotic and narcotic analgesics at less than depressant doses prevented the reaction to phenyl quinone, this suggests a high degree of specificity. The "syndrome" seems not to be associated with intestinal spasm which may be produced by the phenyl quinone injection, since atropine sulfate, adiphenine hydrochloride or dicyclimine hydrochloride, at doses exhibiting marked anticholinergic and antispasmodic activities, failed to prevent reaction. The "syndrome" ceased following administration of antihistaminics (tripelennamine hydrochloride or diphenhydramine hydrochloride) at doses producing stimulation. At one-half the dose producing stimulation, frequency and intensity of the "syndrome" was less than for the controls. It will be recalled that histamine was once proposed as the chemical mediator in cutaneous pain perception. Procaine hydrochloride given orally failed to prevent the "syndrome" at subconvulsive doses. However, intraperitoneal injection of 0.5% solution suppressed the "syndrome" immediately for 4-9 minutes. This clearly demonstrates that the "syndrome" is associated with centripetal impulses which arise following injection

TABLE II. Antagonism to the "Syndrome" of Narcotic Analgesics in Mice.

Drug*	Form administered	ED ₅₀ (C.L., p = .05)† mg/kg	Time of peak effect (min.)
Morphine sulfate	Aqueous solution	1.15 (.88-1.50)	30
Meperidine hydrochloride	<i>Idem</i>	4.00 (3.10-5.20) 6.30 (4.40-9.00)	15 30
Codeine phosphate	"	6.40 (4.78-8.55)	15
Methadone hydrochloride	"	.78 (.57-1.09)	30

* Drugs are admin. subcut.

† C.L. = Confidence limits.

tion of phenyl quinone into the abdominal cavity. Much work (including spinal animals) remains to be done in order to elucidate the mechanism of this syndrome.

Discussion. It is not known whether the "syndrome" is closely associated with pain and the method may also lack some of the critical requirements for an ideal analgesic-testing method as outlined by Goetzl *et al.* (3). However, it has been shown that the "syndrome" caused by a noxious stimulus exerted in the abdominal cavity can be specifically blocked by local anesthetics (administered locally) and by analgesics (administered systemically). Furthermore, there is a good correlation between clinical efficacy of a number of analgesics and their potency as established by this method. In addition, the activity ratios of several narcotic analgesics are in general agreement with the experimental results reported in the literature. Therefore, it seems that the present method would serve best in evaluating, with the greatest clinical predictability, analgesic potency in laboratory

animals.

Summary. A simple method has been designed for evaluating both non-narcotic and narcotic analgesics. The method is based upon the specific antagonism of analgesics to the typical "syndrome" produced by intraperitoneal injection of 2-phenyl-1,4-benzoquinone in mice. The antagonism occurs at doses far below those producing depression or other toxic effects. There is also a good correlation between clinical efficacy of a number of analgesics and their relative potency as established by this method. The endpoint is sharp, and the method is simple and quantitative.

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Received May 31, 1957. P.S.E.B.M., 1957, v95.

Purified Diets for Ruminants.* (23346)

GENNARD MATRONE, H. A. RAMSEY, AND G. H. WISE

Animal Nutrition Section, Department of Animal Industry, N. C. State College, Raleigh

It is established that the greater proportion of the carbohydrates in the diet of ruminants is absorbed as lower fatty acids of which acetic, propionic and butyric predominate (1). It appears, moreover, that on a roughage diet little or no enzymatically digestible carbohydrate escapes ruminal fermentation to the volatile acids (2,3), and that acetic acid is a major tissue metabolite of ruminants (4,5). Thus, it can be reasoned that the peculiarity of ruminant nutrition in contrast to that of the simple-stomach animal may be in the kind of substrate in the diet serving as the primary source of energy rather than in terms of new nutritional entities.

Procedure.[†] The formulas of the experi-

mental diets are presented in Table I. Diet 1 was formulated along the lines of purified diets used for small laboratory animals; diet 2 contained acetic, propionic and butyric acids to simulate the predominant volatile acid end products of ruminal fermentation on a forage diet (6); diet 3 contained cellulose to simulate a roughage diet. Thirteen lambs, 1 to 3 weeks old, were separated from their dams and placed on a regimen of fortified fresh cow's milk (7) administered *via* nipped bottles. At approximately 3 months of age, 12 of these lambs were allotted at random to the experimental diets in a randomized block design; the extra lamb was assigned arbitrarily

* Approved by Director of Research for publication as Paper No. 817 in the Journal Series.

[†] The authors express their sincere appreciation to Mr. F. A. Lane for his care and feeding of the animals.

TABLE I. Purified Diets Used in Investigation.

Components	Diet 1	Diet 2	Diet 3
	%		
Casein	30	30	30
Glucose*	36	24.1	16
Hydrogenated veg. oil†	4	4	4
Corn starch	20		20
Cellulose‡			20
Salts of lower fatty acids§		31.9	
Vitamin mixture¶	5	5	5
Mineral mixture	5	5	5

* Crystalline glucose, Corn Products Sales Co., Norfolk, Va.

† Grateful acknowledgment is made to Buckeye Cotton Oil Co., Cincinnati, Ohio, for Primex B and C (pure vegetable shortening) and alpha cellulose cotton linter pulp.

‡ Ca propionate 4.8%, Na propionate 3%, Na acetate 9%, K acetate 8.5%, Mg acetate 3%, and Na butyrate 3.6%. Grateful acknowledgment is made to E. I. duPont de Nemours and Co., Wilmington, Del., for Ca and Na propionate and to Farmers Cooperative Exchange, Raleigh, N. C., for acetate and butyrate salts.

§ Vit. mixture/100 lb diet: thiamine HCl, 400 mg; riboflavin, 850 mg; nicotinic acid, 1.13 g; Ca pantothenate, 1.42 g; pyridoxine HCl, 570 mg; folic acid, 57 mg; p-aminobenzoic acid, 1.13 g; inositol, 11.35 g; biotin, 11.4 mg; choline chloride, 113.45 g; menadione (2-methylnaphthoquinone), 115 mg; 0.1% vit. B₁₂ (with mannitol), 4.66 g; alpha tocopherol acetate, 570 mg; glucose, 2132 g. Acknowledgment is gratefully made to Merek and Co. for contributing the vitamins.

Admin. 4000 I.U. A and 400 I.U. D/day/100 lb body wt via capsules generously contributed by R. P. Scherer Corp., Detroit, Mich.

|| Mineral mixture/100 lb diet: CaHPO₄, 818 g; KCl, 273 g; NaCl, 239 g; MgSO₄, 204 g; CuSO₄ · 5H₂O, 893 mg; FeSO₄ · 2H₂O, 7648 mg; MnSO₄ · H₂O, 1399 mg; ZnO, 2263 mg; CoCO₃, 9 mg; glucose, 722 g.

¶ Mineral mixture/100 lb diet: trace minerals, Cu, Fe, Mn, Zn and Co, 12 g (See ||); NH₄Cl, 138 g; H₃PO₄ (85%), 226 g; glucose, 1892 g.

to diet 2. The change-over from the basal milk diet to the dry experimental diets was effected in approximately 3 weeks. The lambs were fed individually in pens made of aluminum pipe siding and wooden slatted floors. Lambs receiving diets 1 and 3 were fed *ad libitum*, whereas those receiving diet 2, because of its deliquescent nature, were restricted to the amounts they would consume 3 times a day. The animals were weighed at weekly intervals. Blood samples were taken biweekly, before the morning feeding, from the jugular vein of the experimental animals. The serum of these samples was analyzed for calcium, phosphorus and magnesium by the methods used by Hawkins *et al.*(8) and iron

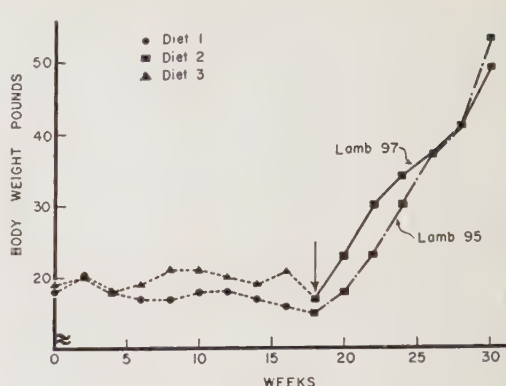


FIG. 1. Responses of 2 lambs to diets 1 and 3, followed by diet 2.

and copper by the methods described by Matrone *et al.*(9). Hemoglobin was determined by the method of Shenk *et al.*(10) and blood glucose by the method of Mendel *et al.*(11).

Results. After 18 weeks on experiment, only the lambs on diet 2 had made appreciable gains (Table II). In view of these results, one lamb on diet 1 and one lamb on diet 3 were changed to diet 2. The response of these animals to diet 2 (Fig. 1) was marked. The average daily gain for these 2 lambs over a 12-week period was 0.41 lb, and the average feed efficiency 3.1 lb of feed/lb of gain.

The 40-week gains of the animals continued on original treatments are shown in Table II. It is apparent that the superior response of lambs receiving diet 2 over those receiving either 1 or 3 was maintained. These data also indicate that the lambs fed diet 3 gained more than those fed diet 1. Feed intake paralleled the gains, *e.g.*, during the last 15 weeks of the 40-week experimental period the aver-

TABLE II. Averages of Initial Weights, 18-Week Gains and 40-Week Gains of Lambs on 3 Purified Diets.

Diet No.	Initial wt, lb	18-wk gain, lb	40-wk gain, lb
1	26	.5 (4)*	6.7 (3)†
2	20	27.0 (5)	68.5 (4)‡
3	20	2.0 (4)	18.3 (3)†

* No. in parentheses = No. of lambs receiving diet.

† One lamb changed to diet 2 (Fig. 1).

‡ One animal died; autopsy revealed gastro-enteritis and extensive passive congestion of both lungs.

age daily intake per lamb was as follows: diet 1, 0.64 lb; diet 2, 1.42 lb, and diet 3, 0.93 lb.

Other differences were also observed. Lambs on diets 1 and 3 ate their own wool in characteristic patterns determined by their ability to reach certain areas of their bodies, whereas the lambs on diet 2 were not observed either eating their own wool or with areas denuded of wool. It was observed, moreover, that the lambs on diet 2 appeared to have a higher than normal respiration rate, which was accentuated on hot days. This effect might have been a contributing factor in the death, during the summer, of the most rapidly growing lamb. Autopsy revealed gastro-enteritis and extensive passive congestion of both lungs. Several times thereafter during the summer, when other lambs on diet 2 developed an excessive rate of respiration, it was reduced by spraying them with water.

The mineral constituents of the serum (calcium, magnesium, phosphorus, iron and copper) and hemoglobin of the blood of all of the experimental animals were within the normal range. Glucose levels of the blood, which averaged 42.6 ± 10.5 , 41.6 ± 10.8 , and 44.6 ± 11.2 mg % for the lambs receiving diets 1, 2 and 3, respectively, were not significantly different.

Discussion. The data that have been presented strengthen the view that the volatile fatty acids play a vital role in ruminant nutrition. The results with growing lambs in this investigation are in accord with supplementary observations with 3 mature sheep, in which weight changes during a 4-month period were as follows: diet 1, -50 lb; diet 2, +11 lb, and diet 3, -16 lb. The higher respiration rate observed in the animals fed the diet containing the salts of the volatile fatty acids may possibly be the result of the higher specific dynamic action associated with the metabolism of acetate(3). Another possibility is that a "subclinical" alkalosis resulted from the high cation content of the diet.

The poor growth of the animals receiving diet 1 was probably due, in part at least, to the changes that occurred in the dietary starch and glucose in the rumen rather than to the inadequacy of the diet. Except for a

lower percentage of fat, diet 1 is quite similar to synthetic milk diets used successfully with young calves by Wiese *et al.*(12) and Blaxter (13). Under our conditions, moreover, lambs fed a fortified milk diet(7) grew in an apparently normal manner to a weight of 80-90 lb. In the experiments cited, however, the diets were fed in liquid form *via* nipples. In such a procedure, presumably the diet bypasses the rumen to a considerable extent and thereby escapes ruminal fermentation. When the diet is fed in the dry form, most of it would be expected to enter the rumen, where the dietary starch and glucose are fermented, probably to lactic acid(1). Although under normal circumstances lactic acid in the rumen is unstable and is fermented further to propionic and acetic acids(1), under heavy starch feeding the lactate may remain in the rumen in considerable concentrations and the pH then may become very acid(14). It is possible that such a sequence of action took place in the rumen of the animals fed diet 1, since its content of starch and glucose is high.

The growth obtained with the lambs fed the cellulose diet was below expectation. One possible explanation of this result may be that the inoculum for a normal rumen flora was absent, since the animals were housed away from older ruminants and had access only to purified ingredients. The study with mature sheep, in which the animal on diet 2 gained 11 lb whereas the animal on diet 3 lost 16 lb, suggests that other factors were also involved, since presumably the sheep started with a normal rumen flora.

Summary. The efficacy of 3 purified diets, each containing a different mixture of energy-yielding constituents, for growth of lambs was investigated. Preliminary results indicate that lambs fed a diet containing glucose and 31.9% of the salts of volatile acids, acetic, propionic and butyric, performed better than lambs on diets containing either starch and glucose or starch, glucose and cellulose.

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Received June 6, 1957. P.S.E.B.M., 1957, v95.

Effect of Ferric Chloride on Mobilization of Accumulated Liver Cholesterol, in Mice.* (23347)

WILLIAM T. BEHER, WILLIAM L. ANTHONY, AND GIZELLA D. BAKER

Edsel B. Ford Institute for Medical Research, Henry Ford Hospital, Detroit, Mich.

Siperstein, Nichols, and Chaikoff(1) successfully prevented hypercholesterolemia in cockerels by addition of ferric chloride to a diet rich in bile concentrate and cholesterol. They based their work on two well-known facts: 1. Cholesterol absorption in the intestinal tract of the rat is hindered if bile is not present(2); and 2. Ferric chloride precipitates bile salts, *in vitro*. In our previous experiment(3), addition of varying amounts of ferric chloride to cholesterol-rich diets did not prevent the accumulation of exogenous cholesterol in mouse liver. Friedman, Byers, and Gunning(4) showed that in rats excess organ cholesterol is converted to bile acids prior to excretion. It has been shown by Hummel(5) that the mobilization of excess liver cholesterol in mice is prevented by dietary bile acids. It would follow from these observations that any substance which would remove bile acids from the intestinal tract, should increase the rate of cholesterol mobilization. This experiment attempts to answer 2 questions: Does the addition of ferric ions to the diet increase rate of liver cholesterol mobilization in mice with elevated liver cholesterol? Do ferric ions reverse effects of dietary cholic acid?

Methods. Approximately 200 albino female Webster strain mice, weighing 15-20 g, were placed on a cholesterol-free diet (C.F.D.) for 2 weeks. The animals were then divided into 2 groups: Group A with 40 mice, and Group B with 160 mice. Group A was continued on the basic C.F.D. while Group B diet was supplemented with 1% cholesterol and 0.5% cholic acid. After a 3-week period on this regime, 7 Group A and 10 Group B animals were sacrificed. As shown in Table I, the supplemented diet resulted in a significant build-up of liver cholesterol in the Group B animals, but in no change in either total lipids or phospholipids. After this build-up period, the remaining Group B animals were divided into 4 groups of approximately 40 mice each and placed on the following diets: Group C received the C.F.D.; Group D received the C.F.D. plus 0.5% cholic acid; Group E, C.F.D. plus 1% ferric ions; and Group F, C.F.D. plus 0.5% cholic acid and 1% ferric ions. At weekly intervals about one-fourth of the mice from each group were sacrificed by decapitation. The livers were immediately excised, drained of excess blood, weighed, and quick frozen. The livers were dried over phosphorous pentoxide, weighed, and extracted with a 1:1 mixture of methanol and chloroform. Aliquots of the extracts were

* This investigation was supported, in part, by grant from Michigan Heart Assn.

TABLE I. Effect of Dietary Cholic Acid and Cholesterol on Liver Lipid Pattern in Mice in 3 Weeks.

Group	Phospho- lipid	Total lipid	Total choles- terol
	%		
A. Normals	7.74±1.2	36.30±6.42	1.76±.32
B. Developed fatty livers	6.60±.55	35.09±3.11	7.69±.89

± numbers are stand. dev.

used for gravimetric determination of total lipids. Suitable aliquots were used for determination of cholesterol by the Schoenheimer-Sperry method, as modified by Sperry and Webb(6), and for lipid phosphorous according to the method of Fiske and SubbaRow (7). The phosphorous values were multiplied by 25 to obtain phospholipid levels.

Results. Table II summarizes the weekly regression period analyses for phospholipid, total lipid, and cholesterol in each group. Values for total lipids show slight variations; however, these are not statistically significant. While in all groups the phospholipid values varied during the regression period, none of these values were significantly different from

those for Group A.

Total cholesterol values are presented in the last column of Table II. It can be seen that the control group (C) regressed to normal (Group A) levels within the 4-week period. A comparison of Groups C and D shows that dietary cholic acid prevented regression of liver cholesterol, and that in fact it caused an increase in cholesterol levels during that period. Dietary ferric ions did not alter the rate of cholesterol regression (Groups C and E). A comparison of Groups D and F indicates that the simultaneous feeding of ferric ions and cholic acid did not reverse the effect of cholic acid alone as observed in Group D. Such a reversal was expected because of the large excess of ferric ions. From the data presented here, it can be concluded that ferric ions do not prevent cholic acid absorption in mice.

Summary. 1. Dietary ferric ions do not increase rate of liver cholesterol mobilization in mice. 2. Mobilization of elevated liver cholesterol is prevented by addition of dietary cholic acid. 3. Evidence is presented which suggests that cholic acid absorption is not

TABLE II. Effect of Ferric Ions and Cholic Acid on Lipid Pattern in Cholesterol Type Fatty Liver.

Group	Time, wk	Phospholipid	Total lipid	Total cholesterol
		%		
A. Normals	1	5.21 $\pm$.75	39.85 $\pm$ 5.60	2.29 $\pm$.41
	2	2.86 $\pm$.67	36.01 $\pm$ 4.28	1.85 $\pm$.31
	3	4.03 $\pm$ 1.06	33.17 $\pm$ 2.97	2.07 $\pm$.25
	4	4.52 $\pm$.68	36.99 $\pm$ 3.01	2.80 $\pm$.93†
C. R*—Controls	1	4.80 $\pm$.70	44.30 $\pm$ 4.84	4.93 $\pm$.85
	2	2.66 $\pm$.77	29.44 $\pm$ 2.62	3.65 $\pm$.60
	3	4.93 $\pm$.78	40.24 $\pm$ 3.92	2.93 $\pm$.96
	4	3.39 $\pm$.96	37.75 $\pm$ 7.36	3.15 $\pm$ 1.51†
D. R—Cholic acid	1	4.85 $\pm$.88	33.97 $\pm$ 3.78	4.81 $\pm$.72
	2	3.63 $\pm$.48	33.91 $\pm$ 4.16	5.19 $\pm$ 1.04
	3	5.86 $\pm$ 1.24	34.36 $\pm$ 4.08	6.54 $\pm$ 1.33
	4	3.10 $\pm$.55	34.08 $\pm$ 3.64	6.30 $\pm$ 1.42†
E. R—Ferrie ions	1	4.95 $\pm$.78	34.01 $\pm$ 2.90	4.17 $\pm$ 1.19
	2	2.85 $\pm$.55	31.73 $\pm$ 4.52	3.02 $\pm$ 1.00
	3	4.22 $\pm$.72	37.14 $\pm$ 4.16	3.40 $\pm$ 1.01
	4	3.85 $\pm$.82	40.50 $\pm$ 4.77	3.57 $\pm$.47†
F. R—Cholic acid and ferric ions	1	3.62 $\pm$.67	32.45 $\pm$ 3.40	7.64 $\pm$ 1.22
	2	3.66 $\pm$.45	35.50 $\pm$ 3.46	5.52 $\pm$.97
	3	4.63 $\pm$.67	30.13 $\pm$ 4.60	4.56 $\pm$ 1.66
	4	4.29 $\pm$.81	31.75 $\pm$ 1.45	4.86 $\pm$ 1.89†

* R = Regression.

† Groups C and A, $P = .65$. Groups C and D, $P < .01$. Groups C and E, $P = .55$. Groups D and F, $P = .13$.

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Received June 6, 1957. P.S.E.B.M., 1957, v95.

Association of Thyrotropin with γ -Globulin in Myxoedema.* (23348)

J. M. MCKENZIE (Introduced by E. B. Astwood)

*Ziskind Research Laboratories, New England Center Hospital, and Department of Medicine,
Tufts University School of Medicine, Boston.*

In human serum, thyrotropin has been stated to be associated with β -globulin(1,2). However, Postel(3) demonstrated that when bovine pituitary thyrotropin was added to human serum, and this solution fractionated by electrophoresis in a starch block, thyro-

tropic activity was present only in the gamma globulin fraction. Using a highly sensitive bioassay for thyrotropin(4), endogenous thyrotropic activity has now been found only in association with gamma globulin.

Methods. The bioassay procedure was



FIG. 1. Identification of a fraction of serum obtained by electrophoresis on a starch block. The gamma globulin fraction (top) and whole serum (bottom) simultaneously electrophoresed on paper at pH 8.6.

* This work was supported in part by grants from Rockefeller Foundation, American Cyanamid Co., and the U.S.P.H.S. The author is grateful to Dr. E. B. Astwood for much helpful advice.

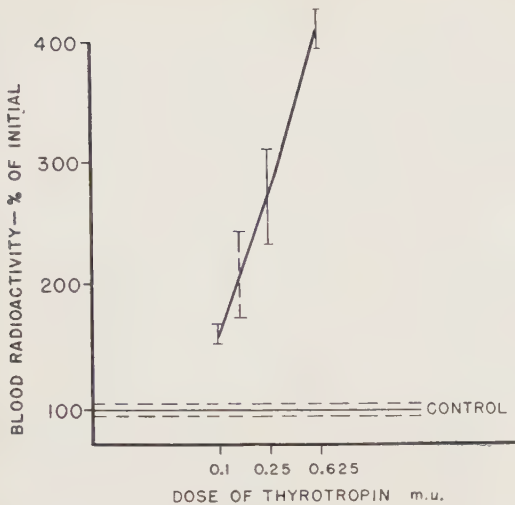


FIG. 2. Assay of gamma globulin from the serum of a patient with myxedema. Mean response $\pm$ stand. error of the mean shown for 6 observations of each dose level of U.S.P. Reference Standard Thyrotropin (continuous vertical lines) and of the unknown (interrupted line). For calculations of the result see text.

adapted from the method of Adams and Purves(5). Twenty g female mice, on a low-iodine diet, were given an intraperitoneal injection of I^{131} followed immediately by a subcutaneous injection of 10 μ g thyroxine. 0.066% Thyroid (U.S.P.) was then added to their diet and the animals used for thyrotropin assays 4, 5, and 6 days later. The radioactivity was measured in 0.1 ml of venous blood, obtained 2 hours after intravenous injection of U.S.P. Reference Standard Thyrotropin or the solution being assayed. This radioactivity was compared with that present before the injection. With groups of 6 mice used in this manner it was possible to detect 0.025 international milliunits (i.m.u.) of thyrotropin and the precision of the assay (λ) averaged 0.25.

Three to 5 ml samples of serum from patients with myxedema were fractionated by the method described by Kunkel and Slater (6), on a block of washed starch suspended

in borate buffer of pH 8.6. After elution of 1-cm segments of the block, the protein distribution was determined by spectrophotometry (λ 278). The appropriate eluates were combined, dialyzed, and lyophilized. They were subsequently redissolved and assayed for thyrotropin content. The identity of the protein fraction was confirmed by paper electrophoresis at pH 8.6, comparison being made with simultaneously electrophoresed whole serum. Separation of gamma globulin was particularly satisfactory by this method (Fig. 1).

Results. Serum fractions from 4 patients with myxedema were assayed. Responses typical of thyrotropin were produced by the gamma globulin fraction in each case. Negative responses were obtained by assay of the other protein fractions. In a typical experiment (Fig. 2) the mean response of the gamma globulin was equivalent to 0.141 i.m.u. thyrotropin. This was obtained with injections of 0.2 ml of a 2.4% solution, obtained from the electrophoresis of 3 ml serum. The whole fraction was dissolved in 1.5 ml saline and this serum was thus calculated to contain 0.302 i.m.u. thyrotropin/milliliter.

Summary. A bioassay technic able to detect 0.025 international milliunits of thyrotropin was developed. Using this technic, fractions obtained from the electrophoresis of serum of patients with myxedema were assayed. Thyrotropic activity was found only in association with γ -globulin.

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Received June 7, 1957. P.S.E.B.M., 1957, v95.

Some Metabolic Actions of Orinase.* (23349)

RUBIN BRESSLER AND FRANK L. ENGEL†

Departments of Medicine and Physiology, Division of Endocrinology, Duke University, Durham, N. C.

The precise mechanisms by which the hypoglycemic arylsulfonylureas exert their action(s) have not yet been fully elucidated. Demonstrations of alterations in several biological parameters have brought postulations of multiple sites of action(1,2,3,4). Among the suggested mechanisms have been: (a) stimulation of insulin secretion; (b) inhibition of glucose output by the liver; (c) inhibition of gluconeogenesis; (d) inhibition of glucagon secretion; (e) inhibition of insulinase. Analysis of published data indicates that no single mechanism is responsible for all observed effects and that several of the postulated mechanisms may be operative(5, 6,7). The evidence supporting a stimulation of insulin secretion is considerable(8,9,10, 11,12,13). However, failure to demonstrate any peripheral effects of insulin with arylsulfonylureas has not been reconciled with this interpretation(14,15,16).

The present studies were designed to derive additional information concerning metabolic responses to arylsulfonylureas. Attention was directed to its action on fasting glycemia and ketonemia in relation to known role of the liver in ketogenesis, and these responses were correlated with effect on liver glycogen. Observations were made on the influence of the drug on accumulation of glycogen in the dorsal interscapular fat pad of the rat because this tissue has been shown to be uniquely sensitive to the action of insulin(17,18,19). The results of these experiments are consistent with a mechanism of action mediated by insulin, but do not constitute proof of such a mechanism.

Methods. Male albino rats of the Vander-

bilt strain, weighing 170-200 g were used. Orinase was administered in doses of 400 mg/kg in 0.5% NaHCO₃ by stomach tube in 4 cc volume. Control animals received equal volumes of the bicarbonate solution. Blood sugars were determined by the photometric method of Somogyi(20). Blood ketones were determined by the method of Werk *et al.*(21). For liver glycogen analyses, rats were anesthetized with nembutal and the entire liver removed. Livers, weighing 3-6 g, were divided. Two portions weighing 300-800 mg each were put in boiling 30% KOH. Another portion weighing 1-3 g was homogenized in cold 10% trichloroacetic acid in Waring blender. Glycogen determinations were done by the method of Carroll, Longley and Roe (22) using the anthrone reagent. Glycogen determinations of the dorsal interscapular fat pad were done as previously described(17), except for final analysis of glycogen(22).

Results. Tables I and II record the effect of Orinase on glycemia, ketonemia and liver glycogen of the 24 hour fasted rat. A marked depression of glycemia and ketonemia occurred within the first hour after Orinase administration. Blood sugar levels remained low and showed no significant rise by the sixth hour. The ketone levels demonstrated a significant rise by the third hour and had returned to pretreatment levels by the sixth hour. When a second dose of Orinase was administered at the sixth hour, the ketone levels could again be depressed without any further changes in blood sugar. Thus, in a group of 10 rats, blood ketone levels fell from 7.5 mg % to 2.6 mg % 2 hours after Orinase and then rose at 6 hours to 7.1 mg %. Two hours after a second dose of Orinase blood ketone values had again fallen to 2.8 mg %. The corresponding blood sugar values were 91, 40, 38 and 41 mg %.

Liver glycogen levels of the Orinase treated group were elevated over those of their re-

*We are indebted to Miss Joan Fredericks for valuable technical assistance and to Dr. C. J. O'Donovan of Upjohn for generous supplies of Orinase.

†Supported by research grants from the American Cancer Society and from the National Institute of Arthritis and Metabolic Diseases, N.I.H.

TABLE I. Effect of Orinase on Blood Sugar and Ketone Body Levels in 24 Hour Fasted Rats.

Time, hr	Blood sugar, mg %			Blood ketones, mg %, as acetone		
	Control	Orinase	P	Control	Orinase	P
0	89 ± 1.8 (54)			7.1 ± .32 (28)		
1		44 ± .7 (11)			2.7 ± .02 (11)	
2	79 ± 2.3 (30)	39 ± 1.5 (49)	<.01	7.4 ± .48 (12)	2.9 ± .02 (36)	<.01
3		31 ± 4.0 (17)			4.3 ± .35 (18)	
5	76 ± 9.4 (16)	37 ± 1.9 (33)	"	7.9 ± .52 (16)	5.1 ± .33 (33)	"
6		43 ± 1.8 (18)			6.8 ± .37 (22)	
15	84 ± 4.3 (10)	78 ± 7.9 (19)	N.S.	7.6 ± .73 (12)	7.5 ± .48 (16)	N.S.

Results expressed as mean ± stand. error.

No. in parentheses refers to No. of animals used.

TABLE II. Effect of Orinase on Liver Glycogen (mg/100 g Liver) of 24 Hour Fasted Rats.

Time, hr	Control		Orinase		P
	KOH extraction	TCA extraction	KOH extraction	TCA extraction	
2	246 ± 39.0 (15)	172 ± 29.8 (14)	482 ± 46.0 (28)	349 ± 40.3 (28)	<.01
5	307 ± 47.2 (14)	206 ± 39.0 (14)	744 ± 91.4 (24)	487 ± 52.6 (24)	"
15	844 ± 154.8 (7)	410 ± 93.3 (7)	777 ± 97.5 (12)	303 ± 33.3 (12)	>.5

No. in parentheses refers to No. of animals used.

Orinase—400 mg/kg p.o.

Difference between 2 hr control and 15 hr control—P >.02 <.05.

spective controls at both 2 and 5 hours. The level at 5 hours was significantly greater than that at 2 hours ($p < 0.01$). At 15 hours the liver glycogen level of the Orinase treated group was not elevated over the 5 hour group or over its respective 15 hour control. The glycogen levels of the controls at 15 hours (39 hour fast) were higher than controls at 2 hours (26 hour fast), $P > 0.02 < 0.05$. The glycogen changes were equally apparent in the KOH and TCA fractions.

Orinase had no effect on accumulation of glycogen in the dorsal fat pad of 24 hour fasted rats 2, 5, or 15 hours after its administration. To conserve space these data are not recorded. Since previous studies on glycogen deposition in the dorsal interscapular fat have shown that more pronounced glycogen elevations could be obtained by refeeding of glucose after a 48 hour fast (17,18,19), the experiment was repeated using these conditions. Rats were fasted 48 hours and divided into 3 groups. One group (16 rats) received Orinase at zero time, another group (12 rats) received subcutaneous injection of 1 unit of NPH insulin at zero time; and one group (12 rats) acted as controls. All these groups were tube fed 2 ml of 50% glucose at 2 hours, 1 ml of 50% glucose at 4 hours and were sacrificed

at 6 hours. Both the insulin and Orinase treated groups had significantly lower blood sugars than the control group at 2, 4, and 6 hours. Insulin promoted deposition of glycogen in the dorsal fat pad (1085 ± 164.0 mg/100 g) while Orinase did not, (654 ± 78.5 mg/100 g), even though blood sugar depression was comparable in each case. The glycogen level in the control group was 637 ± 107.0 mg/100 g.

Mirsky has postulated that stimulation of insulin secretion is an early response to Orinase (23). He points out, however, that the response may be difficult to demonstrate in the fasted animal because fasting decreases the insulin content of the pancreas (6,24). We, therefore, reinvestigated the effect of Orinase on fat pad glycogen in the non-fasted rat. Because an immediate response was being sought, the animals were sacrificed at 3 hours after administration of the drug. One group received Orinase, one group received 1 unit of HGF free regular insulin subcutaneously, and the controls received bicarbonate. All groups received 2 ml of 50% glucose at start of experiment. The glucose was tube fed simultaneously with the Orinase and the bicarbonate to the respective groups. Table III shows that a highly significant increase in

TABLE III. Effect of Orinase and Insulin on the Dorsal Fat Pad Glycogen of the Rat.

Group	Blood glucose in mg %			mg glycogen/100 g	P
	0 hr	1 hr	3 hr	3 hr	
Glucose alone* (8)	131 $\pm$ 3.3	112 $\pm$ 5.7	90 $\pm$ 11.0	212 $\pm$ 29.5	—
" + Orinase† (8)	106 $\pm$ 2.9	64 $\pm$ 5.2	66 $\pm$ 2.5	662 $\pm$ 95.0	<.01
" + insulin‡ (6)	109 $\pm$ 2.6	55 $\pm$ 2.7	33 $\pm$ 3.3	1253 $\pm$ 226.0	"

* Glucose—2 ml of 50% glucose p.o. at start.

† Orinase—400 mg/kg p.o. at start plus glucose as above.

‡ Insulin—One unit H.G.P. free regular insulin inj. subcut. at start plus above glucose.

Results expressed as mean $\pm$ stand. error.

No. in parentheses refers to No. of animals.

adipose tissue glycogen content occurred in both the insulin and Orinase treated groups. This is a clear demonstration of a peripheral response to Orinase.

Discussion. Our experimental data are subject to a number of interpretations, but are not inconsistent with the immediate response to Orinase being mediated by stimulation of insulin secretion by the pancreas. They throw no new light on the mechanism of the prolonged action of the drug.

Although many data previously presented favor an action of the arylsulfonyleureas on stimulation of insulin secretion, the failure to reproduce any of the usual peripheral actions of insulin with these drugs has barred acceptance of this interpretation(25). Mirsky(6), however, has cautioned that if an immediate secretion of insulin is only one of the actions of the drug, it might not be readily demonstrated if the insulin content of the pancreas is reduced when the experiment is carried out. Our study bears this out for it was impossible to demonstrate an increase in glycogen concentration of adipose tissue in the 24 or 48 hour fasted animal following Orinase administration, although insulin itself was effective. On the other hand, when the experiment was repeated in the fed animal, which would be expected to have adequate insulin stores, a prompt increase in adipose tissue glycogen content was noted. To our knowledge, only insulin has been found effective in increasing adipose tissue glycogen(17,18,19) and hence, until some other mechanism is described, this observation may be taken as strong presumptive evidence of an insulin action following Orinase. Krah(26) was unable to detect an *in vitro* effect of the drug on

adipose tissue metabolism.

The combination of hypoglycemia, a fall in fasting ketonemia and an accumulation of liver glycogen point to an hepatic action of Orinase. Other investigators have shown that the drug inhibits glucose output by the liver(27,28,29,30) and there is increasing evidence now that insulin has a similar action(31,32,33). The fall in blood sugar after Orinase is associated with a prompt but transient decline in ketonemia and a second dose of the drug after ketonemia has been reestablished immediately reduces ketone levels again. Insulin has an identical action‡ and it seems probable that this response reflects an action on hepatic ketogenesis(34). If one accepts the growing evidence, reviewed by Dunn *et al.*(33), that insulin does indeed have an action on the liver, the above data with Orinase are again consistent with an insulin-mediated action of the drug. Indeed, in view of the prompt antiketogenic effect of the drug and the absence of a demonstrable action on adipose tissue during fasting, it is conceivable that if insulin is secreted in response to Orinase ingestion, its major action during fasting may be on the liver rather than on the peripheral tissues. In evaluating the action of a drug which has the postulated action of stimulating insulin secretion it must be borne in mind that the response to insulin secreted into the portal vein and reaching the liver first may not be identical, qualitatively or quantitatively, with that of extracted insulin injected peripherally. This consideration may have bearing on some of the discrepancies in published reports concerning Orinase.

It should be emphasized that while our data

‡ To be published.

are compatible with the hypothesis that insulin secretion is stimulated, they certainly do not establish it. There are ample data to indicate that other mechanisms of action of the sulfonylurea drugs must also be operative (35,36,37,38). Further investigation is still necessary to establish the validity and significance of the various postulated actions of these agents.

Summary. 1. The fasted rat is responsive to the hypoglycemic effects of Orinase within one hour. Blood sugar is not restored until the fifteenth hour. 2. Orinase depresses the fasting ketosis of the rat. The effect is a rapid and transient one, but may be reobtained by a second dose of the drug. 3. Orinase causes a rise in liver glycogen of the fasting rat. 4. Orinase causes a deposition of glycogen in the interscapular fat pad of the fed, but not the fasted rat. 5. Similarities between the acute responses to Orinase and the action of insulin are discussed in relation to postulated hepatic and extra-hepatic actions of insulin.

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Received June 14, 1957. P.S.E.B.M., 1957, v95.

Urinary Bladder Sterols.* (23350)

J. S. FINLAYSON,[†] J. L. GAYLOR, AND C. A. BAUMANN

Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison

The ratio of Δ^7 -sterols to cholesterol is much higher in rodent urine than in blood, and preliminary analyses indicated the urinary bladder to contain sterols of the Δ^7 -type (1). The present study deals with the nature of these sterols, the amounts found in various species, their distribution within the bladder, and their syntheses from acetate by bladder slices *in vitro*.

Methods. Bladders were dissected out, cut open, rinsed in cold isotonic saline, and subjected to acetone extraction for 24 hours in a Soxhlet apparatus. The fat-free tissue was then dried and weighed, and the acetone extract analyzed for cholesterol and Δ^7 -sterol (2). For identification and measurement of 7-dehydrocholesterol, the sterol was precipitated with digitonin, the digitonides were split with pyridine(3), and the free sterol was dissolved in ethanol and the absorption spectrum read in a Model 11 Cary spectrophotometer.[‡] For separation of mucosa from the muscle layer, bladders were soaked in N/3 NH_4OH (4) for 10 minutes, rinsed in distilled water, and the gelatinous mucosa scraped off with a dull scalpel. Dog bladders used for incubation with radioactive acetate *in vitro* were taken from beagle pups 7 to 8 weeks old; mouse bladders and skin, from Sutter stock mice 13 to 15 weeks old. After dissection, bladders were rinsed in Krebs-Henseleit bicarbonate buffer (pH 7.4), blotted, weighed, cut into slices and incubated for 3 hours at 37°C with $\text{CH}_3\text{C}^{14}\text{OONa}$ in 50 ml flasks (1 g of tissue/flask) containing 6 ml of buffer (5). The gas phase was $\text{CO}_2:\text{O}_2(1:19)$. Following incubation, the contents of the flasks were combined, saponified with ethanolic KOH and the non-saponifiable fraction

treated with digitonin. The digitonide was split and aliquots of the sterol fraction were examined for 7-dehydrocholesterol, total Δ^7 -sterol, and cholesterol as described above. Carrier Δ^7 -cholesterol was then added, and p-phenylazobenzoyl ("azoyl") esters of the sterols were prepared and chromatographed on silicic acid - Celite columns(6). After development, the columns were cut, the azoyl esters eluted, measured by their absorbance at 450 $\text{m}\mu$ in benzene(7) and aliquots plated on copper planchets from a solution of polyvinylpyrrolidone in chloroform and counted in a thin-window Geiger-Müller counter. In the case of the Δ^7 -sterols from mouse bladder the azoyl esters were saponified and the 7-dehydrocholesterol removed by forming the maleic anhydride adduct(8). Counts remaining in this fraction were then attributed to Δ^7 -cholestenol.

Results. Urinary bladders of mice, rabbits, chinchillas, rats, guinea pigs, dogs, pigs, sheep and cattle were analyzed for cholesterol and fast-acting sterols. Bladders of rodents showed the highest contents of fast-acting sterol, the dog intermediate amounts, and livestock very little (Table I). Guinea pig, chinchilla, rabbit and mouse bladders contained more cholesterol than did those of other species (Table I). As previously reported for rats and mice(1), bladder sterols of all species were unesterified(9).

In rodents and in the dog most (60-84%) of the fast-acting sterol was 7-dehydrocholesterol (Table I). Recrystallized bladder sterol was analyzed for 7-hydroxycholesterol by the method of Sobel *et al.*(10), but in no instance was any of this sterol detected. The other fast-acting sterol in bladder was therefore assumed to be Δ^7 -cholestenol. The major sterol was identified as cholesterol by the melting point of its azoyl ester, the spectrum of its Tschugaeff chromophore prepared by the method of Hanel and Dam(11), and the shape of the curve showing L_{620} $\text{m}\mu$ versus time after treatment with the Liebermann-

* Published with approval of Director of the Wisconsin Agri. Exp. Station. Supported in part by grant from N.I.H., U. S. Public Health Service.

[†] P.H.S. Research Fellow of Nat. Cancer Inst.

[‡] Procedure for analysis and calculation of the amounts of sterols in the mixture found in bladder is described in detail in reference(9).

TABLE I. Sterols in the Urinary Bladder of Various Species.

Species	mg/100 g FFDW $\pm$ S.D.		7D (Δ^7 -Sterol) $\dagger$ $\times 100$	No. samples
	Cholesterol	Δ^7 -Sterol*		
Mouse	1260 $\pm$ 160	142 $\pm$ 20 (129)	69	3
Rabbit	1310 $\pm$ 120	117 $\pm$ 22 (106)	60	3
Chinchilla	1450 $\pm$ 15	79 $\pm$ 10		2
Rat	790 $\pm$ 140	70 $\pm$ 12 (62)	84	6
Guinea pig	1340 $\pm$ 95	54 $\pm$ 7 (49)	61	3
Dog	815 $\pm$ 240	24 $\pm$ 9 (22)	71	5
Pig	895 $\pm$ 20	12 $\pm$ 4 (12)	0	3
Sheep	965 $\pm$ 40	10 $\pm$ 2 (10)	0	4
Ox	685 $\pm$ 120	3 $\pm$ 1 (3)	0	4

Abbreviations: FFDW = fat-free dry wt; S.D. = stand. dev.; 7D = 7-dehydrocholesterol.

* Calculated as Δ^7 -cholesterol; values in parentheses represent mixture of Δ^7 -cholesterol and 7-dehydrocholesterol present in the species.

$\dagger$ Mixture of Δ^7 -cholesterol and 7-dehydrocholesterol.

Burchard reagent(2).

In dogs, pigs, sheep and cattle, analyses of separated mucosa and musculature indicated a 3- to 5-fold concentration of cholesterol in the mucosa compared with the muscle layer (Table II). Δ^7 -Sterol, on the other hand,

TABLE II. Distribution of Sterols within the Urinary Bladder.

Species	Tissue	mg/100 g FFDW $\pm$ S.D.	
		Cholesterol	Δ^7 -Sterol*
Dog	Mucosa	2970 $\pm$ 390	610 $\pm$ 320
	Muscle	930 $\pm$ 105	9 $\pm$ 2 $\dagger$
Pig	Mucosa	5040 $\pm$ 1580	220 $\pm$ 75
	Muscle	915 $\pm$ 30	10 $\pm$ 4
Sheep	Mucosa	3090 $\pm$ 10	60 $\pm$ 15
	Muscle	870 $\pm$ 90	5 $\pm$ 2 $\dagger$
Ox	Mucosa	2220 $\pm$ 330	8 $\pm$ 1 $\dagger$
	Muscle	635 $\pm$ 100	3 $\pm$ 1 $\dagger$

Abbreviations: FFDW = fat-free dry wt; S.D. = stand. dev. for 4 samples.

* Calculated as Δ^7 -cholesterol.

$\dagger$ Values below 10 mg/100 g cannot be regarded as reliable.

showed a concentration gradient of up to 60-fold in this respect. In no case did the Δ^7 -sterol exceed 1.1% of the bladder muscle sterol, values generally being too low to be

regarded as reliable (Table II). The mucosa contributed from 1 to 2% of the fat-free dry weight of bladder(9).

Radioactive acetate was incorporated into both the Δ^7 -sterol and the cholesterol zones of bladder, but much more actively into the former, by a factor of 35 to 150 times (Table III). The actual difference may have been greater still, since high counting companions arising from tissue slice incubations can accompany cholesterol on the azoate column (5). Mouse skin, included for comparison, was only slightly more active as a site of Δ^7 -sterol synthesis than was mouse bladder (Table III). Since little of the Δ^7 -sterol resulting from dog tissue incubations was 7-dehydrocholesterol, no conclusions can be drawn regarding the synthesis of this sterol in dog bladder. In mouse bladder, however, where 7-dehydrocholesterol constituted 64% of the Δ^7 -sterol, specific activities of 7-dehydrocholesterol and Δ^7 -cholesterol were 2700 and 1530 counts/minute/mg, respectively, a ratio exactly equal to that of the amounts of these 2 sterols.

Discussion. In a preliminary communica-

TABLE III. Synthesis of Sterols from $\text{CH}_3\text{C}^{14}\text{OONa}$ by Bladder Slices.

Species	Tissue	Run No.	Counts/min./mg sterol		$\frac{7\text{D}}{\Delta^7} \times 100$
			Cholesterol zone	Δ^7 -Sterol zone	
Dog	Bladder	I	115	6890	0
	"	II	140	4870	12
Mouse	Bladder	III	14	2090	64
	Skin	III	101	2500	0

Abbreviations: 7D = 7-dehydrocholesterol; Δ^7 = total Δ^7 -sterol.

tion Heaton *et al.* reported the presence of 7-dehydrocholesterol in rat bladder(12) and the present results show this compound to exist in the bladders of several other species accompanied by smaller amounts of another fast-acting sterol, probably Δ^7 -cholestenol. In species in which separation of the bladder mucosa and muscle layers was possible, the sterols, and especially the fast-acting sterols were found to be concentrated in the mucosa. This parallels the observation that Δ^7 -sterols are concentrated in the mucosa relative to the muscle of intestine(13) and in the epidermis relative to the dermis of skin(14,15).

The more active incorporation of labeled acetate into Δ^7 -sterols than into cholesterol by bladder slices is also analogous to the situation observed in skin. Both in mouse bladder and in rat skin(5) 7-dehydrocholesterol had a higher specific activity than Δ^7 -cholestenol. 7-Dehydrocholesterol has been found in vagina, submaxillary glands(12), intestine, blood(13), placenta, pancreas(16), ovaries, stomach(17), and preputial and anal glands(18)—all sites which would not be expected to receive ultraviolet irradiation. This distribution has led Glover *et al.* to suggest a biological function for 7-dehydrocholesterol other than its role as provitamin D₃(13). Such a suggestion is strongly supported by our finding that the synthesis of 7-dehydrocholesterol from acetate was more active than that of the other sterols in mouse bladder.

Summary. 1. Urinary bladders of many species were analyzed for cholesterol and "fast-acting" (Δ^7) sterol. Rodents showed the highest content of Δ^7 -sterol, the dog an intermediate level, and livestock very little. In rodents and in the dog 60-84% of the Δ^7 -sterol was 7-dehydrocholesterol. All bladder sterol was unesterified. 2. The sterols were found to be more concentrated in the mucosa of the bladder than in the muscle

layer, the Δ^7 -sterol showing a greater concentration gradient than cholesterol. 3. Tissue slices of dog and mouse bladder incubated with $\text{CH}_3\text{C}^{14}\text{OONa}$ *in vitro* incorporated label into both the Δ^7 -sterol and the cholesterol fraction, but the specific activity of the former was much (35-150X) higher.

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Received June 14, 1957. P.S.E.B.M., 1957, v95.

Increased Concentration of Water in Uterus of the Rat Following Treatment with Relaxin.* (23351)

M. X. ZARROW† AND D. M. BRENNAN

Department of Biological Sciences, Purdue University, Lafayette, Ind.

It has been known for some time that relaxin induces an increase in the water content of the pubic symphysis of the guinea pig(1) and the reproductive tract of the sow(2); and that estradiol increases the water content of the uterus of the immature rat(3). Since a similarity has been shown in the end effect produced by estrogen and relaxin and since relaxin has been shown to act on the uterus of the rat(4) the following investigation was undertaken to determine whether relaxin also influenced water content of the uterus of the rat.

Material and methods. Approximately 470 female rats of the Harvard-Wistar strain were used. The animals were 24 days old at the time of relaxin administration and had been deprived of food for 72 hours prior to treatment with the hormone. All animals were maintained on 10% glucose in drinking water *ad libitum* throughout the 3-day period of food deprivation. This was necessary to insure a consistent uterine water content of approximately 80% in the untreated animals. Relaxin† was dissolved in water and injected subcutaneously in volume of 0.1 ml. In the time study, the rats were killed at definite intervals from 0 to 48 hours following administration of the hormone. In the study on effect of dosage of relaxin on uterine water concentration, all animals were killed 6 hours following administration of the hormone. Immediately after killing by ether, the uterus was quickly removed from the peritoneal cavity and dissected free of adhering fat and mesentery. A longitudinal incision was made in each horn and the uterus gently rolled on bibulous paper to eliminate luminal water. The uterus was then weighed, dried in

vacuum oven at 50°C for 24 hours, reweighed and the water content and dry weight determined.

Results. Approximately 95 animals were used to obtain the curve illustrating the effect of relaxin on the uterus of the rat as a function of time. Each point represents 10 or more determinations and values were secured at zero time and at 3, 6, 9, 12, 18, 24, 30 and 48 hours following a single subcutaneous injection of 50 guinea pig units (G.P.U.) of relaxin. The data reveal a marked rise in total uterine weight of 25 mg to a weight of 35.5 mg at 6 hours after administration of the hormone (Fig. 1). Thereafter the weight of the uterus showed a steady decline to a low of 22 mg at 18 hours and a return to 25.2 mg at 48 hours. The initial and highly significant increase in uterine weight at 6 hours can be accounted for entirely, on the basis of increased hydration. The concentration of uterine tissue water increased from a pre-injection value of 79.8% to 85.3% at 6 hours after injection of relaxin. No significant change in dry weight of the uterus can be noted at this time. The uterine water dropped to 82.2% at 12 hours and 81.5% at 24 hours. At 30 hours after administration of relaxin, the water content of uterine tissue was back to the pre-injection value of 79.8%. A slight drop in the dry weight of the uterus may be observed at 18 to 30 hours, however this was not significant when analyzed by the Student "T" test. Hence, one can conclude that no significant changes occurred in the dry weight of the uterus after treatment with relaxin.

A second experiment was carried out to determine whether a relationship exists between the dosage of relaxin and the concentration of water in the uterine tissue. Again immature rats at 24 days of age were used and treated as previously indicated. The hormone was administered subcutaneously in a single injection and the dosage ranged from 0.05 G.P.U. to 100 G.P.U. All animals were

* Presented at XX Internat. Congress of Physiol., Brussels, Aug. 1956.

† Aided by grant from Ortho Research Fn.

‡ Obtained in the form of a dry powder or water solution (Releasin[®]) through the courtesy of Dr. R. L. Kroc, Warner-Chilcott Laboratories.

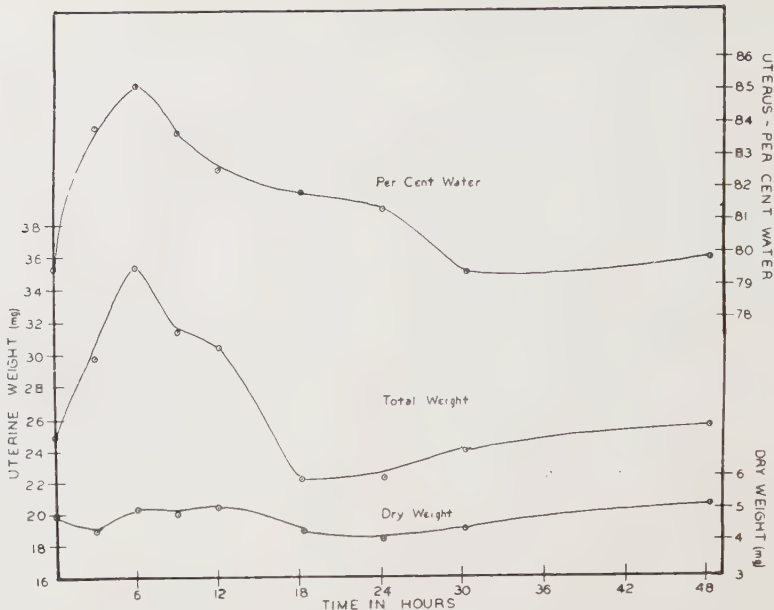


FIG. 1. Changes in % uterine water, dry wt and total wt of uterus following a single inj. of 50 G.P.U. of relaxin. Observations were made at various intervals up to 48 hr.

killed 6 hours following injection of the hormone. No statistically significant changes in water content were noted at dosages of 0.05 to 0.5 G.P.U. of relaxin. The minimum effective dose of relaxin that increased the per cent uterine water of the castrated rat was 1 G.P.U. This caused the uterine water to increase from a control value of 79.8 to 81.4% ($P=0.01$). A maximum response of 84.2% was obtained with 10 G.P.U. (Table I) and further increase in dosage of relaxin to 50 and 100 G.P.U. failed to elicit a significantly greater rise in uterine water content. The data gave a straight line relationship when plotted on a semilogarithmic basis between

the dosages of 0.5 and 10 G.P.U. of relaxin.

Discussion. These data present further evidence for a similarity in the effect produced by relaxin and estrogen and also suggest a significant dissimilarity in the action of the 2 hormones. The present report demonstrates that relaxin can act independently of estrogen in causing an increase in water content of the uterine tissue of the rat, and maximum hydration of the uterus is obtained at 6 hours following treatment with relaxin. This is identical with that seen after estrogen treatment(3). However, the uterine water returned to its pre-injection level within 24-42 hours following injection of relaxin and no subsequent response was noted during this period in either per cent water or dry weight of the uterus. In contrast to this, estradiol will induce a second rise in uterine weight at 30 hours due to an increase in dry weight and represents a true increase in protoplasmic mass. These findings suggest a marked dissimilarity between the 2 hormones and indicate that they may act via different mechanisms. Talbot *et al.*(5) have demonstrated an increase in sodium and a slight decrease in potassium of the uterine tissue following estradiol administration. This would tend to

TABLE I. Effect of Relaxin on Uterine Water Content of the Immature Rat.

No. of rats	Dose relaxin (G.P.U.)	Uterine water (% $\pm$ S.E.)*
91	No Rx	79.8 $\pm$.24
32	.05	80.2 $\pm$.20
32	.1	80.9 $\pm$.20
32	.2	80.4 $\pm$.30
32	.5	80.8 $\pm$.26
32	1	81.4 $\pm$.34
32	2	82.5 $\pm$.32
32	10	84.2 $\pm$.34
32	50	84.4 $\pm$.38
32	100	84.5 $\pm$.28

* S.E. = Stand. error of mean.

indicate that the water shift following estradiol may be due to the action of the hormone on the electrolytes. Present experimentation is now being concluded on the action of relaxin on the electrolytes of the uterus.

It is of further interest that this effect of relaxin on uterine water content is produced without previous sensitization by estrogen. In all actions which have been previously demonstrated, it has been necessary to pretreat the animal with estrogen to obtain an effect. This necessity for estrogen priming is observed in the separation of the guinea pig pubic symphysis, the dilatation of the sow uterine cervix, and inhibition of uterine contractions in the rat.

Conclusions. Relaxin causes an increased concentration of water in the uterine tissue of the immature rat. Maximum hydration was obtained at 6 hours following administra-

tion of the hormone. The minimum effective dose of relaxin is 1 G.P.U. and the maximum response occurs following 10 G.P.U. relaxin. Contrary to the effect seen following estradiol administration, no secondary rise in uterine water content and no increase in dry weight is observed. Pretreatment with estrogen is not necessary for this action of relaxin on the water content of the uterine tissue.

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Received June 17, 1957. P.S.E.B.M., 1957, v95.

Role of Liver on Consequences of Lipid Mobilization.* (23352)

JOSEPH SEIFTER AND DAVID H. BAEDER

Wyeth Institute for Medical Research, Radnor, Pa.

We have reported the isolation of a lipid mobilizer (LM) from the plasma of animals administered cortisone(1-4) and from the posterior pituitary of hogs(5). The greatest sensitivity to the hyperlipemic action of LM occurred in animals which had been inadvertently exposed to minute amounts of potential hepatotoxic agents. This suggested that the liver may play an important role in determining lipid concentrations in the peripheral blood following mobilization of fat from the depots.

Materials and methods. Male and female Wistar strain rats weighing 125-250 g were obtained from the same source as those used in our previous studies(1-5). They were

maintained from weaning on Wayne Chow Checkers supplemented with K penicillin G (4 g/ton) and 0.005% butylated p-hydroxyanisole. Desensitization to the hyperlipemic action of LM was accomplished by changing the diet to Warner Dog Chow which did not contain added antibiotics or antioxidants. Sensitivity was reestablished by restoring the Wayne diet. LM was either from the same batch used previously(1-4) or from a new batch. Bleeding and chemical analysis of the blood samples were by the methods previously described(1-4). Determination of liver cholesterol and fatty acids was by a modification of the Baruch and Chaikoff method(6) as furnished by Kaplan and Cohn. Lipid P in the liver was determined by the method of Zilverschiedt *et al.*(7). Liver glycogen was determined by the method of Seifter *et al.*(8). Hepatectomy was performed in one stage under sodium thiopental anesthesia (20 mg/kg). The hilus of the liver was exposed by a midline incision, ligated with No. 1 braided

* We are grateful to Dr. A. Kaplan and C. Cohn of Michael Reese Hospital for making available to us their method for determination of liver lipids; to R. Gurin, E. E. Livingston, Jr., and R. Reifsnyder for technical assistance, and to W. Bechmann, J. Dachiw, J. Howitz and M. Klenk for chemical determination of plasma and liver lipids.

TABLE I. Effect of LM on Lipids in Liver (%) and Plasma (mg %) 2 Hr after 5 mg/kg IV. 10 rats/series.

Week	Diet	Liver fasted, LM		Plasma fasted, LM		Liver fasted, no LM		Plasma fasted, no LM	
		CH	FA	CH	FA	CH	FA	CH	FA
0	Wayne	1.9	3.4	202	400				
1	Warner	3.4	3.4	143	174				
2		4.6	4.9	82	102				
3		4.8	4.9	64	96				
4		4.4	4.2	62	84	.6	1.2	56	80
5	Wayne	4.3	4.1	60	85	.9	1.8	64	86
6		2.2	3.9	74	90	1.8	2.6	60	87
7		1.2	3.7	200	374	3.0	3.2	62	86

CH = Cholesterol. FA = Total fatty acids.

silk and excised leaving a small stump. This procedure left the vena cava and hepatic portal vein intact. Closure was with continuous silk sutures and 2 hours later the surviving rats were injected intravenously with test material. Only 10 to 20% of rats survived the 4 hours necessary for the experiment. These survivors presumably represent animals with sufficient anastomoses to systemic circulation.

Results. The data in Table I demonstrate the effect of LM on concentration of lipids in liver and plasma of fasted rats during desensitization and resensitization. The largest accumulation of lipids in liver as a result of fasting occurred in rats exposed longest to the Wayne diet. Two hours after intravenous injection of 5 mg LM/kg sensitized, fasted rats had an elevation of total cholesterol and total fatty acids in plasma and a decrease of cholesterol in liver. As desensitization proceeded LM produced a progressive decline in plasma cholesterol and fatty acids and an increase in accumulation of cholesterol and total fatty acids in liver. Resensitization resulted in reappearance of the hyperlipemia and decrease in liver cholesterol.

The data in Table II demonstrate the effect of LM on concentration of lipids in liver and plasma of sensitized and desensitized rats. Fasted, sensitized rats had significant hyperlipemia following injection of LM. Fasted, desensitized rats had questionable elevation of plasma cholesterol and total fatty acids. The lowest concentration of liver lipids occurred in desensitized rats not receiving LM.

Fasting caused questionable increase of liver lipids in desensitized rats but a significant increase in sensitized ones. Injection of LM caused elevations of liver lipids which were greater in fasted, desensitized rats. Liver cholesterol in fasted, sensitized rats was considerably less following injection of LM than in the group receiving no LM.

The data in Fig. 1 demonstrate the effect of LM on concentration of lipids in the liver and plasma of sensitized rats at various intervals following intravenous injection of 5 mg/kg. Each point is the mean of 10 rats. The liver values are in % and the plasma values in mg %. Except for the immediate increase in total fatty acids of liver the injection of LM caused a decrease in the concentration of all lipid components in the liver. Restitution towards normal values began in the 4th hour

TABLE II. Effect of LM on Liver and Plasma Lipids in Sensitized and Desensitized Rats. (2 hr after 5 mg/kg IV.)

Condition	No.	Liver (%)		Plasma (mg %)	
		CH	FA	CH	FA
Fasted sensitized, LM	10	1.8	3.6	324	668
Fasted desensitized, LM	10	4.6	4.9	82	102
Nonfasted sensitized, LM	10	2.8	3.4	62	86
Nonfasted desensitized, LM	10	1.2	3.1	61	90
Fasted sensitized, no LM	20	3.1	3.8	58	84
Fasted desensitized, no LM	10	.6	1.2	56	80
Nonfasted sensitized, no LM	10	1.9	2.7	56	82
Nonfasted desensitized, no LM	10	.4	1.0	52	78

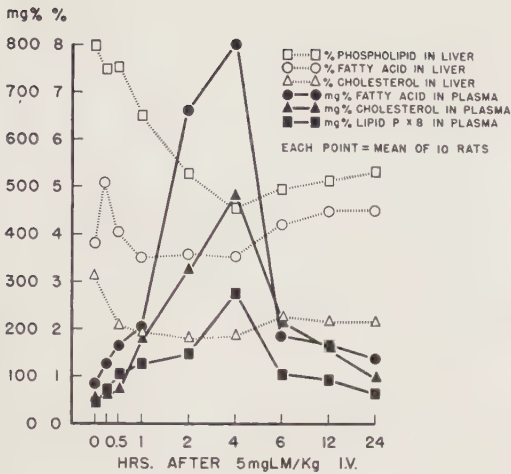


FIG. 1. Effect of LM on lipids in liver and plasma of sensitized rats.

after injection. Coincidental with the fall in liver lipid values there was a marked increase in all plasma lipids which reached their peak concentration in the 4th hour after injection and then fell to almost normal values within the next 2 hours. The data in Table III demonstrate the effect of hepatectomy on response of sensitized and desensitized rats to intravenous injection of 5 mg LM/kg. The operative procedures had no significant effect on the plasma lipid values. Injection of LM into either sensitized or desensitized rats resulted in a marked elevation of the total fatty acids in the plasma but had no effect on either the cholesterol or lipid P. In contrast, injection of LM into sensitized intact rats caused an elevation in the three plasma lipids.

Discussion. The most significant observation was that LM elevated plasma fatty acids in hepatectomized rats whether fasted, nonfasted, sensitized or desensitized, and that hypertriglyceridemia was not associated with corresponding elevations in cholesterol and lipid P. These findings demonstrate that LM mobilized triglycerides from the fat depots and that the liver determined the subsequent concentration of lipids in the blood. In the absence of liver there was hypertriglyceridemia. Liver containing adequate glycogen (4%) either retained or metabolized mobilized lipid in sensitized or desensitized rats. Liver depleted of glycogen (0.09%) handled the fat

load adequately in nonsensitized rats but not in sensitized ones and hyperlipemia resulted. The liver was the source of the excess cholesterol and lipid P in the hyperlipemia as indicated by the data in Fig. 1. The gradual restoration of liver lipids may reflect redeposition from the circulation after the peak effect of LM had passed. The data in Tables I and II suggest that sensitization by administration of potential hepatotoxic agents consisted of producing "subclinical" fatty liver. Sensitized rats had high lipid content in liver and greater accumulation of lipids in response to fasting indicating that they did not metabolize a fat load as well as those desensitized. LM caused elevation of total fatty acids in liver of sensitized, desensitized, fasted or nonfasted rats indicating that it mobilized fat from the depots.

Summary. (1) The role of the liver on the consequences of lipid mobilization from the depots by LM was investigated in sensitized and desensitized rats. (2) Sensitization to the hyperlipemic action of LM involved administration of minute amounts of potential hepatotoxic agents which produced "subclinical" fatty liver as determined by chemical analysis. (3) Desensitization to the hyperlipemic action of LM occurred when the potential hepatotoxic agents were removed from the diet. (4) The liver in desensitized or fed sensitized rats was capable of handling the mobilized lipid without consequent hyperlipemia. (5)

TABLE III. Effect of Hepatectomy on Plasma Lipid (mg %) Response to LM. (2 hr after 5 mg/kg IV.)

Treatment	No. of rats	CH	FA	LP
S + IV NaCl	20	58	86	5.2
S + LM (fasted)	20	398	402	12.6
S + sham hepatectomy + IV NaCl	20	71	98	6.9
S + ligated vena cava + IV NaCl	20	68	94	5.8
S + hepatectomy + IV NaCl	20	70	92	5.0
S + hepatectomy + LM (nonfasted)	20	68	430	5.1
DS + hepatectomy + LM (nonfasted)	6	64	306	5.2

CH = Cholesterol. FA = Total fatty acid. LP = Lipid phosphorus. S = Sensitized. DS = Desensitized.

Fasted, sensitized rats were not capable of handling the fat load and hyperlipemia resulted. (6) The source of the cholesterol and lipid P in the hyperlipemic rats was the liver. (7) LM actively and immediately mobilized triglycerides from the depots and this action did not depend upon sensitization or fasting.

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Received June 17, 1957. P.S.E.B.M., 1957, v95.

Effect of Specific Activity on Distribution of Radiolutecium (Lu^{177}).^{*} (23353)

WILLIAM M. CHRISTOPHERSON, GRANVIL C. KYKER, H. F. BERG, AND
MARSHALL BRUCER (With technical assistance of Johanna Meyer)

*Departments of Pathology and Surgery, University of Louisville School of Medicine, and the
Medical Division, Oak Ridge Institute of Nuclear Studies[†]*

In previous studies on effectiveness of radiolutecium (Lu^{177}) for irradiating lymph nodes in the dog, the possibilities of this rare earth were demonstrated(1,2). Certain difficulties, however, greatly limited the effectiveness of the isotope. With both tracer and therapeutic amounts, irradiation was selectively delivered to the regional lymph nodes draining the mammary gland of the dog. The tissues at the site of local injection tolerated large amounts of the isotope and general body distribution was well below a dangerous level for the animal. The major problem in previous experiments was that often there was not homogeneous distribution of the particles within the lymph node, hence irradiation effects were patchy and often large areas of the nodes remained undamaged. This difficulty was also encountered when Au^{198} was similarly studied(6).

It seemed to us that the amount of rare-earth carrier might influence homogeneity of particle distribution and therefore distribution of irradiation effect. Two factors might be expected to play a role in such a study. The first is that high carrier doses

(low specific activity) of radioactive rare-earths tend to remain at site of injection, whereas low carrier or carrier-free doses (high specific activity) show a greater tendency to be mobilized away from site of injection(3). If we desire to damage the regional lymph nodes of a given area, and to prevent local irradiation damage at site of injection, the material with high specific activity would seem to be advantageous. The other problem concerns the total mass of rare-earth particles available to saturate a lymph node. In previous studies we had injected up to 131 mc of Lu^{177} into the mammary gland of a dog without producing consistent homogeneous radiation effect. In any given node considerably more total concentration of radioactivity was present than was necessary to produce the desired effect; on the other hand, the nodes contained "cold" spots. Dilution of the radioactive particles by additional nonradioactive material would therefore be a possible approach to even dispersion in lymph nodes and their more nearly complete destruction with much lower curiage. This study was undertaken to determine the over-all effect of the specific activity of Lu^{177} on its intranodal pattern. A mixed carrier of stable lutecium and stable yttrium was used in the preparations of low specific activity. The heterogeneous

^{*} This work was supported by a grant from the Damon Runyon Memorial Fund for Cancer Research.

[†] Under contract with the United States Atomic Energy Commission.

carrier was based on previous study of this and of other combinations of rare-earth elements as carrier for the same radioisotope (4):

Method. Two groups of female dogs were used. From 22 mc to 35 mc of Lu^{177} was injected into the right superior mammary gland of each animal. Two weeks later the animals were autopsied. Specimens from site of injection, regional lymph nodes, distant lymph nodes, marrow, liver, spleen, lungs, kidneys, heart, and other selected organs were obtained. Counts for radioactivity were made from a portion of each specimen by means of a wet-ashing method and end-window Geiger-Muller tube. Lymph nodes and other selected organs were examined histologically and autoradiograms were made.

Group A consisted of 5 dogs that received lutecium 177 in preparations of low specific activity. The high level of carrier contained both lutecium and yttrium and specific activity was expressed as millicuries of the radioisotope per mg of total carrier. An average preparation of this type was produced by activation of 30 mg of lutecium oxide (99.9%) in the graphite reactor at Oak Ridge and processing the activated oxide according to the radiochemical procedure described previously (1). The yield was ordinarily from 100 to 120 millicuries of radiolutecium in 10 ml of an isotonic solution of lutecium chloride and sodium chloride at pH 3.5 to 4.0. The dose for one dog was prepared by mixing a portion of the radioassayed solution with a slightly acid standard solution of yttrium chloride (pH 4.0, 60 mg of yttrium per ml) and diluting so that 10 ml contained 30 to 35 millicuries of radiolutecium and 150 mg of yttrium. The combined concentration of lutecium and yttrium was approximately 0.16 molar of which 8% was due to lutecium. The specific activity of 3.3 mc of Lu^{177} per mg of stable lutecium was reduced roughly to 0.25 mc per mg of total carrier by addition of yttrium.

Group B consisted of 12 dogs that received radiolutecium in preparations of high specific activity. The procedure for production of these preparations was designed to include a minimum amount of carrier. The oxide of high purity was first converted to a standard

solution of lutecium nitrate in dilute nitric acid. An aliquot of this solution containing 2.0 mg of lutecium was placed in a small quartz ampoule, evaporated slowly to dryness in an oven at 100°C , and sealed *in vacuo*. The nitrate was activated for 3 weeks in the highest available flux (1.5×10^{13} neutrons $\text{cm}^{-2} \text{sec}^{-1}$) of the low intensity testing reactor (LITR) at Oak Ridge. The activated nitrate was repeatedly extracted with 0.3 ml portions of 1.0 M hydrochloric acid until 98 to 99% of the radioactivity was obtained in 1.2 to 1.5 ml of the acid. The acidic solution was partially neutralized and adjusted in volume to give an isotonic solution at pH 3.5 to 4.0. The yield of radioisotope was ordinarily 100 ± 25 millicuries per mg of stable lutecium. The final concentration of lutecium was approximately 5×10^{-4} molar and the specific activity was roughly 300 times that of the preparations of low specific activity.

Results. When a previous experiment consisting of 8 dogs receiving from 24 to 131 mc of Lu^{177} was used as a base line for comparison, the results in Group A left much to be desired. The site of injection did not tolerate the high-carrier material and severe reactions were the rule. Three of the 5 animals developed large ulcers at site of injection. The other 2 showed evidence of severe acute radiation damage to the skin, subcutaneous tissue, and muscle. Obviously a large amount of radioactivity remained at site of injection. This was further reflected in the axillary, latissimus dorsi, and intercostal lymph nodes. These are the nodes that drain the superior mammary gland, and those that one would expect to accumulate the bulk of the isotope. This had been true in previous experiments with Lu^{177} (1) and Au^{198} (5). Whereas counts for radioactivity were comparatively high in the regional lymph nodes, concentration of the isotope and radiation effect was spotty. The isotope tended to form a sludge in the afferent lymphatic spaces and only rarely showed diffuse concentration in a given lymph node. The sludge could readily be seen in histological sections stained with H. and E. The particulate matter was surrounded by extensive foreign body reaction; for the most part the rest of the node remained undamaged. (Fig.

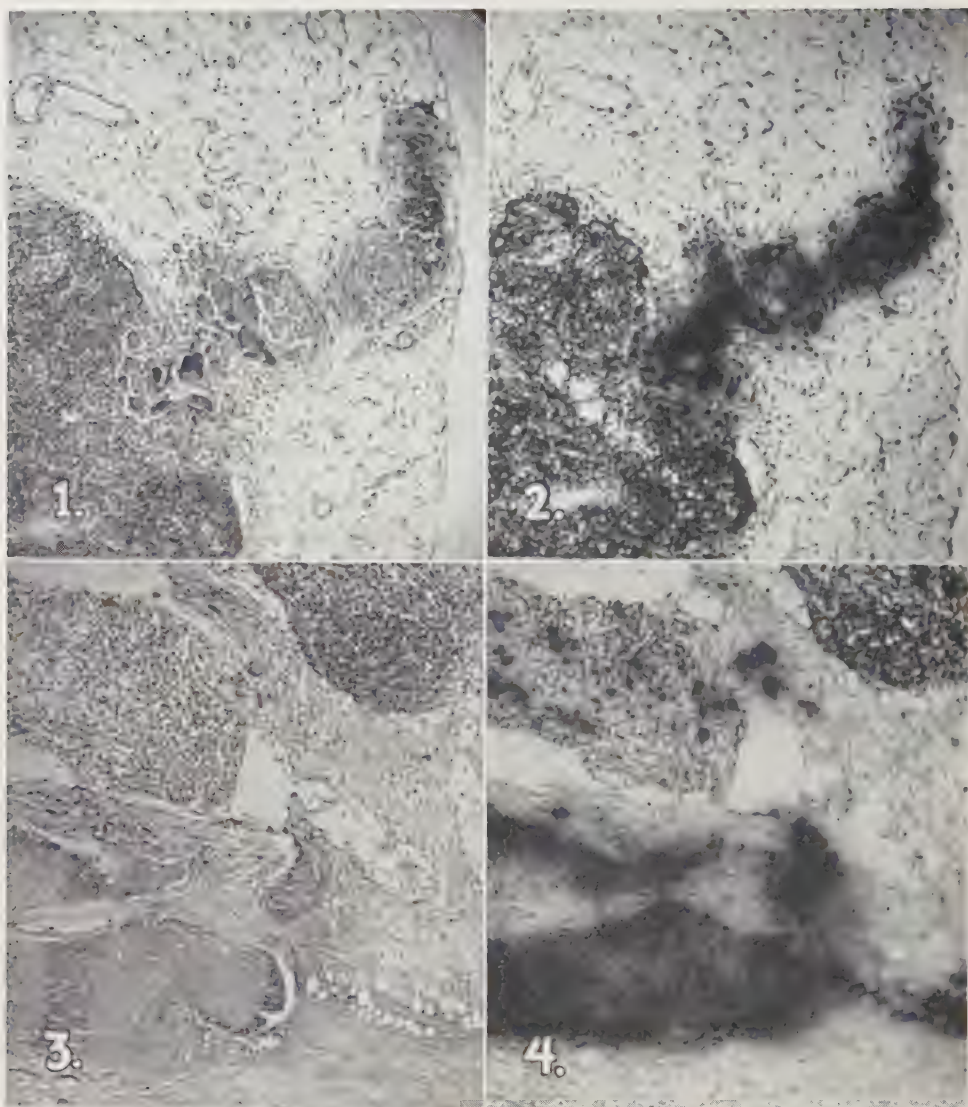


FIG. 1. Right axillary lymph node obtained 2 wk after inj. of 22 mc Lu^{177} into right superior mammary gland. The isotope has formed a sludge in peripheral sinus and perinodal lymphatic vessel.

FIG. 2. Autoradiogram of node illustrated in Fig. 1. Radioactivity limited to the sludge.

FIG. 3. Right latissimus dorsi lymph node showing sludge of isotope in perinodal fat.

FIG. 4. Autoradiogram of node shown in Fig. 3. Note distribution in lymphoid tissue, with high conc. in vascular space.

1 and 3). Autoradiograms showed that intense radioactivity was limited to the areas of sludge (Fig. 2 and 4). A rare node showed good distribution of radioactivity; however, in no instance were all the draining nodes effectively irradiated. No appreciable activity was present in the other organs of the body.

The radiation effect in the regional lymph

nodes of the 12 dogs in Group B was very much better than that obtained in Group A animals. None of the dogs showed severe reaction at the injection site. Slight edema and acute inflammation were present, but no appreciable radiation necrosis was found. Radioactivity counts were high in the lymph nodes draining the site of injection and there

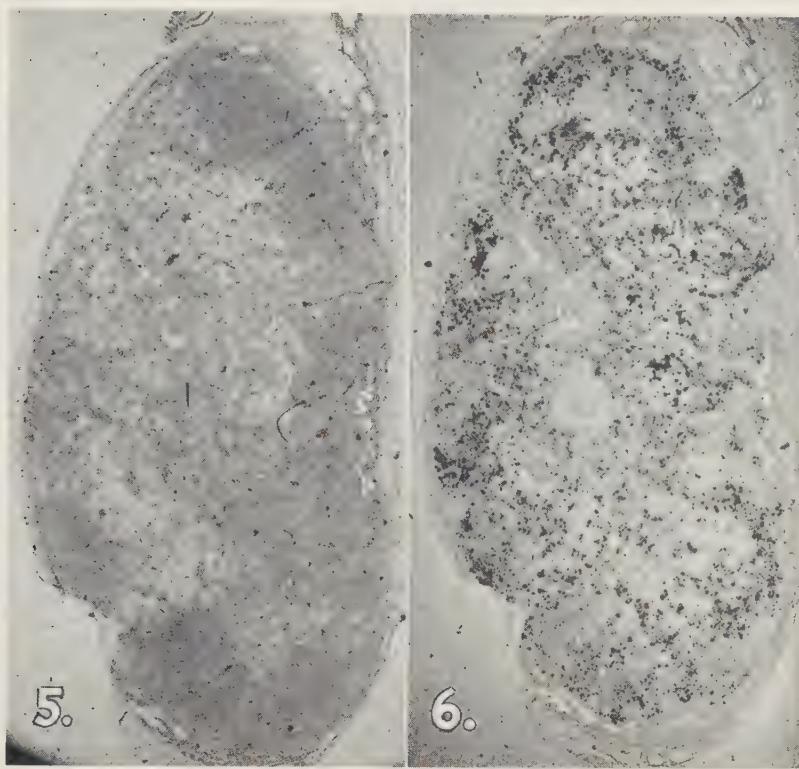


FIG. 5. Right axillary lymph node after inj. of low carrier (high specific activity 2) Lu^{177} . Destruction of node is complete.

FIG. 6. Autoradiogram of lymph node in Fig. 5. Uniform distribution of activity.

was no appreciable general body distribution in such organs as the bone marrow, spleen, or liver. Histological sections and autoradiograms of the organs showed radioactivity and radiation damage limited to the right latissimus dorsi, right axillary, and intercostal lymph nodes. On occasions there was slight activity in the right cervical nodes, but never any radiation necrosis. The lymph nodes on the left side of the body served as controls and showed neither activity in the autoradiograms nor radiation changes. In 3 of the 12 dogs the regional lymph nodes were destroyed. (Fig. 5 and 6). Seven other animals showed partial destruction of some of the nodes with spotty distribution in others. (Fig. 7). In two animals the concentration of the isotope and intranodal distribution was no better than in the Group A animals.

Discussion. The avidity of lymph nodes for radioactive colloids has led to the investigation of their potentialities for selectively ir-

radiating a set of regional lymph nodes in the dog. The colloidal behavior of rare earths at body pH, and the radiation characteristics of Lu^{177} in particular, make it a theoretically useful agent. The great obstacle, however, seems to be the nonhomogeneous distribution of particles within a given set of nodes, even when large amounts of the isotope are used. Since Lu^{177} is chiefly a beta-particle emitter with relatively few low-energy gamma radiations per disintegration, homogeneity of particles must be obtained before one can hope for generalized nodal distribution. When particles of radioactive lutecium are closely packed, more than enough radiations are emitted to destroy the tissue. Greater dispersion of particles was not obtained by mixing with a large chemical dose of nonradioactive particles. The additional inactive rare earth tends to form a sludge blocking the afferent lymphatics thus preventing the isotope from circulating through the lymph node.

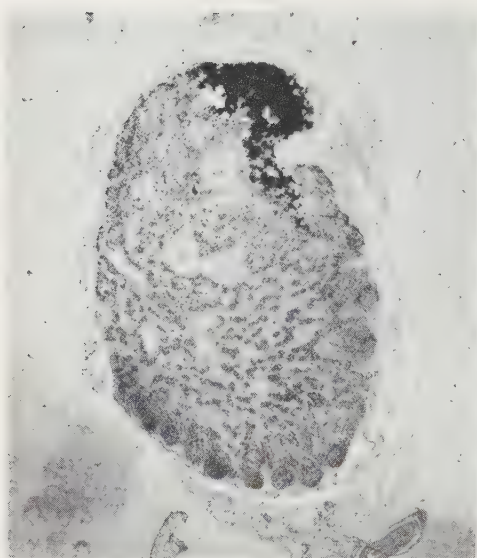


FIG. 7. Focal concentration of radioactivity in lymph node.

Perhaps chemical toxicity plays a part in this phenomenon. It would also seem possible that the radiations play a role in sealing off the afferent lymphatics thus preventing general distribution of the isotope throughout the node.

On the other hand, carrier-free Lu^{177} (high specific activity) is more readily mobilized from the site of injection and incarcerated in the regional lymph nodes. Its distribution within the lymph nodes is much more homogeneous and its resultant radiation effects are more generalized. Intranodal distribution, however, is often uneven and therefore irradiation is inadequate. Certainly the specific activity of the isotope plays a role, then, in

the ultimate pattern of distribution; but unless other presently unknown factors can be controlled the problem of homogeneous intranodal distribution remains unsolved. The compound in these studies was lutecium chloride. Preliminary work indicates that certain lutecium complexes might be more effective.

Summary. When Lu^{177} is injected into the mammary gland of the dog it travels via the lymphatics to the regional lymph nodes. It is concentrated in the lymph nodes and irradiates them. No appreciable amount of radioactivity is accumulated in other organs. A study of the influence of the specific activity of Lu^{177} on its intranodal distribution indicates that a much more effective pattern can be obtained with carrier-free Lu^{177} . High-carrier doses on the other hand produce severe radiation damage at site of injection and ineffective amounts are concentrated within the lymph nodes.

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Received June 25, 1957. P.S.E.B.M., 1957, v95.

Fate of Intravenously Administered Glycerol.* (23354)

D. B. ZILVERSMIT AND ESTHER L. MCCANDLESS

Department of Physiology, University of Tennessee, Memphis

Although glycerol is one of the most widely used pharmaceutical products very little work has been done on the fate or metabolism of intravenously administered glycerol solutions. Perhaps this is due to the fact that early investigators found aqueous glycerol quite hemolytic when given by vein(1). However, this was shown to result from the fact that glycerol penetrates the red cells very rapidly and thus does not exert much osmotic action across the red cell membrane. Deichmann (1) and Miner and Dalton(2) have reviewed the toxicity of glycerol solutions and concluded that glycerol given intravenously is not very toxic when administered in isotonic saline.

In our own studies on intravenous fat emulsions we became interested in the use of glycerol as a dispersing medium for oil and lecithin. We were able to prepare an anhydrous fat emulsion in this manner which could be diluted with 5% glucose to furnish an acceptable high caloric fluid for intravenous use. In the present study we have investigated the disappearance from blood and the urinary excretion of glycerol administered in aqueous solution by vein.

Methods. Mongrel dogs maintained on Purina chow were used for this study. Glycerol solutions were prepared for injection by diluting USP glycerol with 5% aqueous dextrose or 0.9% aqueous NaCl. Plasma glycerol was determined by the method of Lambert and Neish(3) on an aqueous dilution of the supernatant after the addition of 9 ml 10% trichloroacetic acid to 1 ml plasma. Urine glycerol was determined directly. Both urine and blood values were corrected for formaldehydogenic materials present in pre-injection samples.

Results. In previous experiments with dogs infused with intravenous fat emulsions containing 10 to 20% glycerol it was noted that

the secretion of urine increased markedly during the 24 hr period following injection. This agrees with previous observations that glycerol might act as an osmotic diuretic(1,2). In the first experiments of this series we measured the disappearance of intravenously injected glycerol from the bloodstream and its appearance in urine. Dog A (Table I) received 12 g glycerol dissolved in about 50 ml isotonic saline. During the first 30 min. after injection the plasma glycerol level stayed above 2 mg/ml and the urine contained 2.5 g glycerol. After 1 hr, however, when the plasma levels reached 1 mg/ml or below, the amount of urinary glycerol fell to negligible quantities. Thus in the next 2 dogs when the administered dose was reduced to 6 g, plasma levels as early as 5 min. after injection

TABLE I. Plasma Glycerol Concentrations and Total Urinary Glycerol at Various Intervals after a Single Intravenous Dose of Glycerol.

Dog	Dose, g	Plasma glycerol		Urine glycerol	
		Time after inj., min.	mg/ml	Time interval, min.	g
A 10.5 kg	12	30	2.08	0- 60	2.500
		60	1.19	60-120	1.330
		120	1.25	120-180	.351
		180	.35	180-270	.086
		240	.23	270-330	.032
		300	.15		
		360	.13	0-330	4.300
B 10 kg	6	5	.600	0- 60	.250
		10	.552	60-120	.158
		15	.413	120-240	.048
		33	.149	240-360	.024
		60	.095		
		120	.111		
		220	.027		
C 11.4 kg	6	360	.035	0-360	.480
		5	1.33	0-120	.476
		11	1.02	120-270	.120
		17	.82	270-420	.002
		30	.54		
		65	.21		
		135	.15		
		225	.13		
		310	.19		
		375	.19	0-420	.598

* This work was supported in part by a grant from Abbott Laboratories, North Chicago.

TABLE II. Fate of Glycerol in Chronically Infused Dogs.

Dog	Wt, kg	Glycerol infused, g/day	Urine vol, ml/day	Urine glycerol, g/day	Increase in serum glycerol during infusion, mg/cc	Remarks
GP	9	75	1207 $\pm$ 55*	32.7 $\pm$ 1.9	7.3 $\pm$.5	Infused for 21 days. Convulsed on 15th day.
GQ	8.5	58	1039 $\pm$ 51	27.4 $\pm$ 4.2	7.7 $\pm$.6	Infused 16 days. No convulsions.
GL	10.9	75	1305 $\pm$ 53	42.2 $\pm$ 4.3	8.1 $\pm$.7	Infused for 3 days, convulsed. After 1 mo rest, infused for 4 days and convulsed again.

* Stand. error.

tion were down to 0.6 and 1.3 mg/ml and there appeared to be very little excretion of glycerol in the urine.

The next set of experiments was concerned with the fate of glycerol administered in large quantities by daily intravenous infusions. Three dogs received 58-75 g glycerol daily as a 20% solution in isotonic glucose by intravenous drip over a period of 1 hour. The results on these animals are summarized in Table II. Blood glycerol concentrations were determined immediately before, and again at the end of the infusion period. The initial values may have measured the presence of formaldehydogenic materials other than glycerol and in any case were less than 0.2 mg/ml. Twenty-four hour specimens of urine collected during preinfusion periods were less than 200 ml. Infusion of glycerol increased this volume 5-6 fold and it is thus obvious that the administered glycerol solution which amounted to approximately 300 ml exerted a powerful diuretic effect. Immediately after each infusion the dogs were very thirsty and drank a large quantity of water. The amount of glycerol contained in the 24 hr urine specimens was determined daily and appeared to be rather constant throughout the experiment. The 3 animals excreted 44, 47 and 56% of the administered glycerol in the urine and thus utilized for metabolic purposes no more than half of the administered dose.

Large doses of glycerol were administered in this study in order to throw some light on one of the toxic manifestations previously observed, *viz.* tremors and convulsions. These signs appeared in most dogs receiving more

than 5 g glycerol per kg body weight, but only after repeated injections of the glycerol. In several animals it appeared that the occurrence of convulsions sensitized the animal to subsequent infusions. For example, one dog convulsed after 9 daily glycerol infusions of 6.8 g/kg(4). When given a rest period of 3 days the animal convulsed on the first injection. After another 2 day rest convulsions occurred again after a single injection. During the rest periods the animal appeared quite normal.

At the time it was postulated that during a prolonged course of injections an animal might store glycerol by an inability to excrete or metabolize glycerol and thus gradually build up a convulsive level. This, however, was not found to be the case in the present study. Urinary excretions did not show any trend to diminish over the injection period. Nor did the daily preinjection blood levels show any tendency to increase throughout the experiment. To make an additional test on this point, 2 animals were given on 4 separate occasions during the course of injections a single dose of 6.3 g glycerol and the disappearance from blood was determined. The results are shown in Fig. 1. Dog GP was tested prior to infusion and again after 7, 13, and 20 infusions. All disappearance curves fell within a narrow range. Indeed dog GP was tested in this manner on the day prior to the severe convulsions and again at the end of the infusion series without any noticeable changes in disappearance rates. Dog GQ showed only one disappearance curve suggesting some retention but this was not apparent

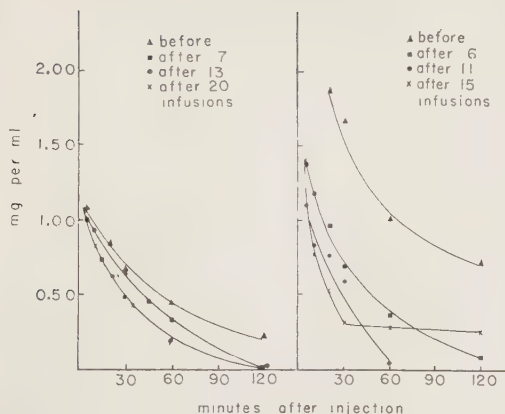


FIG. 1. Glycerol disappearance curves. Left, dog GP; right, dog GQ.

on later tests and this animal never convulsed. The third animal in Table II was chosen for its earlier demonstrated sensitivity to glycerol. About a year before these tests this animal had received 6.5 g glycerol/kg/day and convulsed on the 19th day; after a rest period of one week in a second series of infusions of 5.8 g/kg/day the dog convulsed after the 10th infusion. Nine months later convulsions occurred once on the third day after infusing 6.9 g/kg/day and again after a one month rest on the 4th day of infusing the same amount. This animal did not demonstrate any retention of glycerol, as evidenced by adequate urinary excretion, and preinjection

blood levels on the days of convulsion of 0.06 and 0.08 mg/ml which were even lower than the average preinjection levels in other dogs. Thus there appears to be no evidence that during a course of glycerol infusions the animal develops an inability to dispose of this material from the bloodstream.

Summary. Intravenously administered glycerol rapidly disappears from the bloodstream. Single injections of 6 g glycerol did not produce excessive urinary excretion of glycerol but 12 g led to excretion of one-third the dose. Daily infusions of large doses of glycerol produced marked polyuria and in some animals led to tremors and convulsions. These disturbances were not caused by accumulation of glycerol in the blood.

The authors gratefully acknowledge the technical assistance of Mrs. Shirley Williams.

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Received June 25, 1957. P.S.E.B.M., 1957, v97.

Production in Mice of Large Volumes of Ascites Fluid Containing Antibodies. (23355)

J. MUNOZ* (Introduced by Wayne W. Umbreit)
Merck Institute for Therapeutic Research, Rahway, N. J.

Studies of mouse antibody have been limited by the difficulty of producing serum of high titer as well as by the limited amount of blood that can be obtained from each mouse. Recently Lipton, Stone and Freund(1) have shown that a high titer of antibody can be obtained in the serum of rats immunized with antigens mixed in Freund's adjuvant, and

Stone(2) has obtained similar results in mice. However, the problem of collecting large amounts of serum in mice still remains. Recently it has been observed that mice injected intraperitoneally with antigens mixed with Freund's adjuvant develop large amounts of peritoneal fluid that has been found to contain specific antibody in high concentration. Due to the practical importance of this observation for investigation of mouse antibody as well as the study of anaphylactic reactions

* Present address: Department of Bacteriology, Montana State University, Missoula.

TABLE I. Volume of Ascites Fluid Produced and Qualitative Determination of Antibodies.

Days after last antigen inj.		Mouse No.							
		1	2	3	4	5	6	7	8
14	Vol	10	14	5	2	2	0.5		
	Ring test	4+	4+	2+	2+	+	4+		
25	Vol	13	13	10	4	2	3	5	8
	Ring test	4+	4+	2+	—	+	4+	4+	4+

in mice using homologous antibody, a detailed report of these preliminary observations is given here.

Materials and methods. Swiss Webster female mice weighing from 16-18 g from our own laboratory were used. Each mouse received 2 injections given 2 weeks apart of 1 mg of either bovine serum albumin[†] or 5 times recrystallized egg albumin mixed in Freund's adjuvant (3). The adjuvant was made as follows: Two parts of paraffin oil ("Vaccinol," obtained from the Pennsylvania Refining Co., Butler, Pa.) containing 2 mg (dry weight) of killed *Mycobacterium phlei* cells per ml were mixed with 1 part of Arlacel A (Atlas Powder Co., Wilmington, Del.), and autoclaved at 15 lb for 20 min. After cooling, 3 parts of sterile adjuvant were added to 2 parts of a saline solution of antigen containing 10 mg/ml. The mixture was emulsified by shaking. Each mouse received two 0.25 ml injections given 2 weeks apart of antigen-adjuvant mixture and was then allowed to develop ascites for at least 3 weeks.

The peritoneal cavity was tapped with an 18 gauge needle and the fluid was allowed to flow into a test tube. On many occasions the needle was removed and peritoneal fluid continued to flow out of the needle puncture site. The fluid was tested for presence of antibodies by the ring test method using a saline solution of the corresponding antigen containing 0.1 mg/ml.

Results. Typical results obtained after 2 injections of 1 mg of 5 times recrystallized egg albumin in adjuvant are given in Table I, where volumes of peritoneal fluid as well as results of ring precipitin test for each of 8 different mice tapped on 2 different occasions are given. The volume of fluid collected on the first tapping, 2 weeks after the last injection

of the antigen-adjuvant mixture, ranged from 0.5 ml to 14 ml/mouse with 2 mice having no significant amount of fluid at this time. The second tapping 25 days after the second immunizing injection yielded volumes ranging from 2 ml to 13 ml/mouse, and all mice had some fluid. A third tapping, not included in Table I because the identity of individual mice was lost, was performed 40 days after the second immunizing dose was done on 5 mice and the fluid obtained ranged from 5 ml to 18 ml/mouse. As can be seen from Table I, only one fluid failed to show presence of antibodies to egg albumin. The total amount of fluid collected from these 8 mice in a period of 9 weeks (3 tappings for most mice) was 143.5 ml for an average of 17.9 ml of fluid per mouse.

Since all the mice do not develop ascites the following experiment was performed to establish the frequency with which mice receiving 2 injections of Freund's adjuvant-antigen mixture develop this condition. Fifty mice received 1 mg of crystalline bovine serum albumin in adjuvant per injection. Two weeks after the second injection mice were tapped and fluid volumes recorded. Of 50 mice, 38 or 76% gave from 0.5 ml to 13 ml of fluid each. In another group of 50 mice receiving egg albumin instead of bovine serum albumin the number of mice giving significant amounts of fluid was somewhat smaller. This latter group was tapped on 3 different occasions, and the results obtained are summarized in Table II. On the first tapping 25 mice gave from 1 ml to 15 ml each for a total of 180 ml and an average of 7.2 ml/mouse. On the second tapping, again 25 mice gave from 1 ml to 14 ml each for a total of 156 ml of fluid and an average of 6.2 ml/mouse. During last tapping 16 mice gave from 3 ml to 23 ml each, for a total of 156 ml and an average of 9.7 ml/mouse. From these mice a to-

[†] Purchased from Armour & Co., Chicago, Ill.

TABLE II. Number of Mice Developing Marked Ascites of 50 Injected.

Day after last inj.	No. mice tapped	Total vol of fluid collected (ml)	Vol range (ml)	Avg vol of fluid/mouse tapped
14	25	180	1-15	7.2
25	25	156	1-14	6.2
40	16	156	3-23	9.7

Total vol collected 492.

tal of 492 ml of peritoneal fluid has been collected. The mice still appeared healthy after the third tapping. Most of the fluids gave intense precipitation with egg albumin. Relatively few mice failed to respond with production of antibodies in the peritoneal fluid. A pool of the fluids from the first tapping was found to contain 213 μ of antibody nitrogen/ml, as determined by the quantitative precipitin technic(4).

Discussion. The findings here reported may prove to be of great value for investigations of mouse antibody as well as anaphylaxis using homologous antibody. These studies have previously been complicated by the limited amounts of serum that one can obtain from a mouse. Use of peritoneal fluid as a source of antibody would overcome this obstacle.

Fig. 1 shows a mouse that developed marked distension of the abdomen compared to one of its mates of the same age that failed

to give any appreciable amount of ascites. Before tapping this mouse weighed 52 g of which 28 g was due to ascites fluid. This tremendous accumulation of fluid does not occur in all mice, but usually 2 or 3 of 10 mice do give 10-15 ml each. Mice do not seem to be adversely affected by removal of such large amounts of liquid and this material, after clotting and clearing by centrifugation, is not toxic to other mice. Since there are relatively few cells in this ascites fluid as compared to blood, little volume is lost after breaking the clot that forms and centrifuging at 4,000 rpm for $\frac{1}{2}$ hour.

As little as 0.05 ml of peritoneal fluid containing 10 μ of antibody nitrogen has been sufficient to produce passive anaphylactic deaths in pertussis-treated mice. This points out the importance of using homologous antibody in such studies, since a much greater amount of rabbit antibody is required(5). These studies will be reported later.

Summary. Mice injected intraperitoneally with either egg albumin or bovine serum albumin mixed in Freund's adjuvant develop large amounts of peritoneal fluid containing antibodies to the antigen injected. As much as 28 ml of fluid has been collected from a single mouse on a single tapping. Most mice produced from 0.5-14 ml of fluid each. About 50% of the mice respond with accumulation of fluid in the peritoneum.

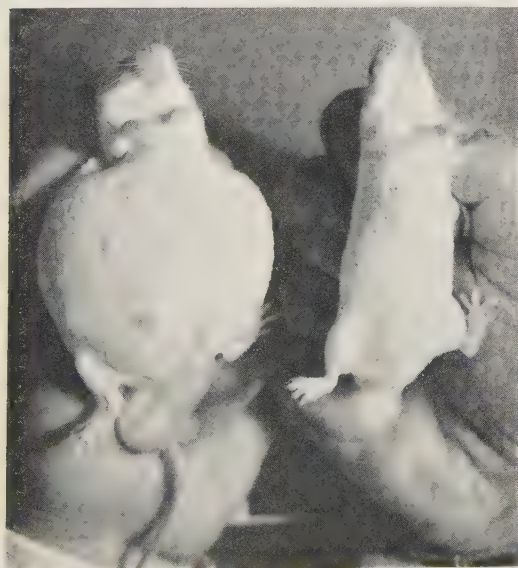


FIG. 1. These 2 mice, of same age, received 2 inj. of adjuvant-antigen mixture. The one on the left developed marked ascites (28 ml of fluid) while the one on the right did not accumulate any fluid.

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Received June 25, 1957. P.S.E.B.M., 1957, v95.

Ultracentrifuge Concentration of Poliovirus and Effect of Calf Serum and Gelatin. (23356)

SAMUEL BARON (Introduced by G. A. Hottle)

National Institutes of Health, Division of Biologics Standards, Bethesda, Md.*

A primary objective in establishment of a poliomyelitis vaccine safety testing program has been to develop methods for detection of the possible presence of live poliovirus in large volumes of poliomyelitis vaccine. Testing of a concentrated poliomyelitis vaccine offers the possibility of combining the facility inherent in manipulating small volumes with the poliovirus sensitivity of the monkey kidney tissue culture system. Although a number of physical and chemical methods have been used for concentrating poliovirus, ultracentrifugation offers a practical and simple procedure involving minimal manipulation or additions of compounds foreign to a biological product. An investigation of the effectiveness of poliovirus concentration in the ultracentrifuge was undertaken because of paucity of data on ultracentrifuge concentration of infectious tissue culture fluids in contrast to the more carefully studied behavior of infected tissue extracts (1,2,10). Results of the present study indicate that poliovirus sedimented from experimental vaccine or Medium 199 has the undesirable quality of forming a loose aggregate easily disturbed by fluid movement. Addition of small amounts of calf serum or gelatin to the system results in formation of a firm pellet which contains most of the virus.

Material and methods. Type I (Mahoney), Type II (MEF₁), and Type III (Saukett) strains were obtained in monkey kidney tissue culture fluid from Dr. Fred Stimpert, Parke, Davis and Co., Detroit, Mich. They were passaged 4 or 5 times in monkey kidney tissue culture and stored at -20°C or in a solid CO₂ chest. Trypsinized monkey kidney tissue cultures were used. These were grown in roller tubes, 2-ounce prescription bottles or 1-liter bottles with either lactalbumin hydrolysate medium or Medium 199(3,4), each containing 2% calf serum. These sera were deter-

mined to be free of poliovirus inhibitors by roller tube titration of virus in the presence of 4% serum. Maintenance medium was the same as the growth medium except that only 1% calf serum was used. Virus titers were determined initially with roller tubes using 5 tubes per either 3.2 fold or 10 fold dilutions in medium. Endpoints were calculated as the number of 50% tissue culture infectious doses (TCID₅₀) per 0.5 ml of inoculum(5). Subsequently plaque titrations in stoppered 2-ounce tissue culture bottles were used(6,7) and the results expressed as plaque-forming units (PFU) per 0.2 ml. To simulate a residually infected vaccine whose behavior in the ultracentrifuge could be studied, commercially prepared poliomyelitis vaccine or Medium 199 was used to dilute infectious virus from original titers of 10^{7.0} TCID₅₀ per 0.5 ml to as low as 10^{0.5} TCID₅₀ per 0.5 ml. These fluids, at pH 7.0 to pH 7.6, were centrifuged in a Spinco model "L" Ultracentrifuge. A No. 30 rotor was used. Thirty-three ml stainless steel centrifuge cups with aluminum caps were filled to capacity with fluid containing virus and centrifuged at 30,000 rpm (78,000 times gravity). To minimize convection currents, the chamber temperature was set to -20°C in order to maintain the fluid at 4°C. Deceleration was allowed to proceed without application of the brake. Various fractions of supernatant were collected by gentle aspiration through the center hole in the centrifuge tube cap with the use of a syringe and side hole 14 gauge needle submerged to a predetermined depth. Any material remaining attached to the cup wall was worked free with a rubber policeman and recovered with three 1.0 ml rinses of medium. This suspension was designated as a sediment. In later studies, a pipette was found to be more convenient for resuspension of the sediment than the rubber policeman and gave equal virus recovery. Fractions, without

* N.I.H., Public Health Service, U. S. Dept. of Health, Education and Welfare.

TABLE I. Distribution of Type II Poliovirus, Added to Poliomyelitis Vaccine, after Ultracentrifugation.

Exp. No.	Time centrifuged (hr)	% poliovirus* in			Total TCID ₅₀		
		Supernatant Upper 31 ml	Lower 2 ml	Sediment	Recovered from contents of centrifuge cup	Present in control held in refrigerator	Calculated from dilution
1	2	26	60	14	10 ^{2.4}	10 ^{2.4}	10 ^{2.9}
2	2	25	62	13	10 ^{6.7}	10 ^{7.1}	10 ^{7.3}
3	2	40	52	8	10 ^{2.7}	10 ^{2.9}	10 ^{3.0}
3	3	0	77	23	10 ^{2.6}	10 ^{2.9}	10 ^{3.0}
4	4.3	5	90	5	10 ^{2.4}	10 ^{2.3}	10 ^{1.9}

* Expressed as % of total TCID₅₀ recovered from contents of centrifuge cup.

clarification, were assayed for virus content immediately or after storage at 4°C overnight. Gelatin was prepared as a 6% stock solution, heated to 80°C for 20 minutes and filtered through an S-1 Seitz-type filter pad.

Results. Sedimentation of virus from vaccine or culture medium. Experiments were conducted to determine effectiveness of ultracentrifugation for concentrating MEF₁ poliovirus in serum-free Medium 199 and in commercially prepared vaccine. Diluted virus suspensions, designed to simulate an incompletely inactivated vaccine, were centrifuged for 2 to 4.3 hours at 78,000 times gravity. The upper 31 ml of supernatant, lower 2 ml of supernatant, and sediment were removed separately and assayed simultaneously with an uncentrifuged control aliquot kept in a centrifuge cup at 4°C during the experiment. Results of these titrations, expressed as percentage of the virus recovered in supernatant fractions and sediment, and as total virus content, are shown in Table I. The yield of the virus in supernatant plus pellet was compared with the total virus derived from the control and with that calculated from the initial virus dilution. Virus recovery in experiments with small and large amounts of virus closely approximated the expected result. This indicates that there was no loss or inactivation of virus in the concentration procedure. Although most of the added virus was concentrated in the lower 2 ml portion of the supernatant, it was not adherent to the cup wall and thus was not collected with the sediment. Increased time of sedimentation did not significantly increase virus content of the sediment but did increase the fraction of virus in

the lower supernatant. In experiments in which virus was diluted in Medium 199 without serum, rather than vaccine, in only 2 of 12 instances was the virus concentrated satisfactorily in the sediment. It was concluded that poliovirus added to vaccine or Medium 199 could not be satisfactorily concentrated in the sediment with the existing method.

Effect of calf serum on concentration of virus from vaccine or medium. In previous work done in connection with virus stability, it had been noted that when calf serum was present in the suspension a firm small brown pellet appeared following centrifugation, but did not form in the absence of serum. Thus it appeared to be due to the serum. It was considered possible that a "calf serum pellet" might enmesh sedimented virus and hold it to the cup wall. On this basis the virus suspension was centrifuged for 5 hours in the presence of 5% calf serum. Analysis revealed 96% of the virus in the pellet, no virus in the upper 31 ml supernatant, and 4% of the virus in the lower 2 ml supernatant. In this instance, addition of calf serum resulted in a pellet which contained almost all the poliovirus. In other experiments similar results were obtained whether the serum was heated at 56°C for 30 minutes or was unheated. To determine the minimal amount of serum required to cause virus adherence to the cup wall, 3 or 4 hour centrifugations were done with 0% to 4% calf serum. Results shown in Table II indicate that a minimum of 1% calf serum in 33 ml of fluid results in adequate concentration. This is correlated with the observation that a definite pellet was not observed in cups with less than 1% calf se-

TABLE II. Effect of Varying Concentrations of Calf Serum on Type II Poliovirus Distribution after Ultracentrifugation for 3 or 4 Hours.

% calf serum	% poliovirus* in	
	Supernatant	Sediment
.0	36	64
.0	37	63
.0	75	25
.015	19	81
.06	43	57
.25	39	61
1.0	0	100
1.0	1	99
2.0	0	100
4.0	0	100

* Expressed as % PFU of total recovered from contents of centrifuge cup.

rum.

Studies described above were done using MEF₁ strain of Type II poliovirus. It was found that Types I and III could be concentrated under similar conditions. The time required to concentrate the virus in the serum pellet was determined by centrifugation for varying periods. The results of experiments with all 3 types of poliovirus are illustrated in Table III. They indicate that maximal concentration in the pellet occurred between 2 and 3 hours of sedimentation when at least 1% calf serum was present.

In previously studied systems some remixing of partially sedimented material usually occurred during deceleration(8). This was caused by the tendency of the fluid to continue rotation. More rapid deceleration increased the tendency. To minimize this effect, all early centrifugation experiments were conducted without application of the brake. To determine whether braking would interfere with concentration of poliovirus in the serum pellet, aliquots of virus in Medium 199

TABLE III. Effect of Time of Ultracentrifugation on Sedimentation of Poliovirus in the Presence of Calf Serum.

Time (hr) centrifuged	No. of experiments averaged	% poliovirus* in	
		Supernatant	Pellet
2	4	23	77
3	5	.5	99.5
4	8	2	98
5	1	4	96
8	1	3	97

* Expressed as % of total TCID₅₀ recovered from contents of centrifuge cup.

TABLE IV. Effect of Calf Sera, Containing Inhibitors to Poliovirus, on Concentration by Ultracentrifugation.

Calf serum	Total virus* in		Total virus* recovered from contents of centrifuge cup
	Super-natant	Sediment	
None	4.2	3.8	4.3
2% No. 6	2.3	3.8	3.8
2% " 7	<2.2	3.8	3.8
2% " 4	2.6	2.7	3.0

* Expressed as log₁₀ PFU.

with 1% calf serum were centrifuged for 3 hours with and without rapid deceleration through application of the brake. In repeated experiments over 95% of the virus was recovered from the pellet under both conditions. This indicated that virus once incorporated into the calf serum pellet is not readily resuspended by disturbances due to braking. It was also determined that decantation of supernatant gave the same good virus recovery from the pellet as did aspiration of supernatant.

Other materials which result in satisfactory concentration into a pellet. The presence of inhibitors to poliovirus in occasional lots of calf serum would be expected to decrease the quantity of virus which could be recovered when these sera were used. Such an effect occurred with the 3 calf sera shown in Table IV. This led to a search for a material which would result in satisfactory concentration and also be free of inhibitors. So that a rational choice of possible substitutes could be made, a study was undertaken to determine which component of calf serum was responsible for the effect. It was found that dialyzed serum retained its effectiveness, indicating that the activity was associated with the protein portion. Paper electrophoretic analysis of calf serum, serum supernatant, and serum pellet,[†] indicated that various globulin components were concentrated into the pellet. This suggested that large protein molecules might have the same effect as calf serum. Some of the materials studied for this property are

[†] Done by Dr. Aaron Cooper of the Division of Biologics Standards, N.I.H., Public Health Service, U. S. Dept. of Health, Education and Welfare.

TABLE V. Effect of Various Additives on Concentration of Poliovirus by Ultracentrifugation.

Additive	Total virus* in		Total virus* recovered from contents of centrifuge cup
	Super-natant	Sedi-ment	
None	4.7	3.1	4.7
Bovine fibrinogen .3%	2.3	3.7	3.7
" γ -globulin .3%	2.5	3.8	3.8
Bacto hemoglobin .3%	3.3	4.1	4.1
Gelatin .3%	2.5	4.5	4.5
Horse serum 3%	4.1	3.1	4.1
Rabbit " 3%	4.2	3.7	4.3
Control titer			4.4

* Expressed as log₁₀ PFU.

shown in Table V. Gelatin (Nutritional Biochemicals Corp.) was chosen for further study because it resulted in satisfactory concentration into a discrete pellet and was free of inhibitors to poliovirus. The other materials were eliminated either because they did not result in satisfactory concentration or because they were inhibitory to poliovirus. Table VI summarizes an experiment designed to determine minimal concentration of gelatin required satisfactorily to concentrate poliovirus into a pellet. It can be seen that 0.015% concentration was minimal. It should be noted from the total virus recovered that gelatin is free of inhibitors to poliovirus.

Concentration of virus preparations partly inactivated by formalin. Under conditions described above live poliomyelitis virus can be effectively concentrated from medium or vaccine to which it has been added. To investigate the possibility that virus incompletely inactivated by formaldehyde might behave differently, experimental poliomyelitis vaccines containing residual live virus were sedimented for 3 hours in the presence of calf serum or gelatin. In repeated experiments, effective concentration of virus was obtained. This indicates similar sedimentation behavior of untreated and formalin treated poliovirus and suitability for use in a vaccine safety test.

Discussion. It has been shown that a large fraction of poliovirus sedimented from serum-free fluids generally is not collected with the sediment but rather with the supernatant. In the presence of added calf serum or gelatin the virus is concentrated in a pellet. It seems

unlikely that calf serum exerts this effect through an increased rate of virus sedimentation, because comparison of Tables I and III indicates that virus is as readily sedimented in the absence as in the presence of serum. Another possible interpretation is that the effect of calf serum is mediated through increased viscosity as has been demonstrated with sucrose(8). This seems unlikely because the calculated change in viscosity induced by 1% calf serum is very small. An explanation which seems to be supported by the data is that a calf serum or gelatin pellet is able to associate with sedimented poliovirus and prevent dispersion by firmly binding it to the cup wall. This would explain the observation that sedimented virus collected at the base of the centrifuge cup, but was firmly adherent to the cup wall only in the presence of calf serum or gelatin. The interpretation assumes that virus sedimented from serum-free fluids forms a loose sediment which is easily disrupted as the supernatant is collected. Additional support for this view comes from experiments in which diminishing amounts of calf serum were used. When the quantity of serum became too small to form a visible pellet, the system simultaneously lost the ability to satisfactorily concentrate virus into the pellet.

Silverberg(9) observed that a 20% mouse brain suspension of Lansing Type II poliovirus was concentrated satisfactorily by ultracentrifugation while a 1% suspension was poorly concentrated. When 10% normal

TABLE VI. Effect of Diminishing Amounts of Gelatin on Concentration of Poliovirus.

% gelatin (final conc.)	% virus* in		Total virus† recovered from contents of centrifuge cup
	Super-natant	Sediment	
.06	4	96	4.2
.06	1	99	4.3
.06	12	88	4.4
.015	3	97	4.5
.004	57	43	4.3
.001	88	12	4.2
.00025	81	19	4.4
.0	77	23	4.3
.0	67	33	4.0

* Expressed as % of total PFU recovered from contents of centrifuge cup.

† Expressed as log₁₀ PFU.

mouse serum was added to the 1% suspension, good concentration occurred and was associated with the appearance of a discrete pellet. The results of this study did not permit conclusions as to the mechanism involved and the variable factors in the system were not further studied.

A disadvantage of calf serum for concentration is the too frequent presence of inhibitors to poliovirus. One alternative was to substitute sera from other species. Results with horse and rabbit sera indicated that these sera at 3% concentration lacked the ability to form a similar enmeshing pellet. Electrophoretic studies of calf serum pellets showed that they were mainly composed of the larger serum molecules. This suggested that other large protein molecules would form such a pellet. A study of some readily available proteins showed that gelatin (Nutritional Biochemicals Corp.) could form a poliovirus-enmeshing pellet and was free of inhibitors.

The data presented demonstrate that reproducible concentration of poliovirus can be accomplished by sedimentation at 78,000 times gravity in the presence of 2% inhibitor-free calf serum or 0.06% gelatin. Such concentrated virus can be used for chemical, physical and serological studies as well as safety testing of larger volumes of poliomyelitis vaccines in a simplified, practical manner.

Summary. Studies were undertaken to determine the efficiency of concentration of poliovirus from tissue culture fluids in the ultracentrifuge. Under suitable sedimentation conditions most of the virus was concentrated

to the lower portion of the centrifuge cup, but in the absence of calf serum was not present in a sediment adherent to the cup wall. Addition of calf serum resulted in formation of a firm, discrete pellet attached to the centrifuge cup wall which contained more than 95% of the virus. Further experiments indicated that the activity was associated with the globulin protein fraction of calf serum and that gelatin had a similar effect. Studies varying the amounts of calf serum and gelatin and duration of ultracentrifugation indicated that 2% calf serum or 0.06% gelatin and 3 hours centrifugation are minimal for effective concentration of poliovirus when sedimented at 78,000 times gravity in the number 30 rotor of the Spinco model "L" preparative Ultracentrifuge.

The author would like to acknowledge the valuable suggestions made by Dr. Robert Rustigian during the initial stages of this study, and the technical assistance of Mr. George Gardner.

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Received June 25, 1957. P.S.E.B.M., 1957, v95.

Postirradiation Creatinuria in *Macaca mulatta*.^{*} (23357)

GEORGE M. KRISE, CLYDE M. WILLIAMS, AND DONALD R. ANDERSON

Radiobiological Laboratory, The University of Texas and the United States Air Force, Austin, Texas

The authors have observed a profound cre-

atinuria in rats immediately following large doses of radiation(1). Subsequently it was found that 2 dogs irradiated with 400 and 500 rep of X-ray, respectively, did not excrete ex-

^{*}This work was supported in part with funds provided under Contract AF 41(657)-149 and the School of Aviation Medicine.

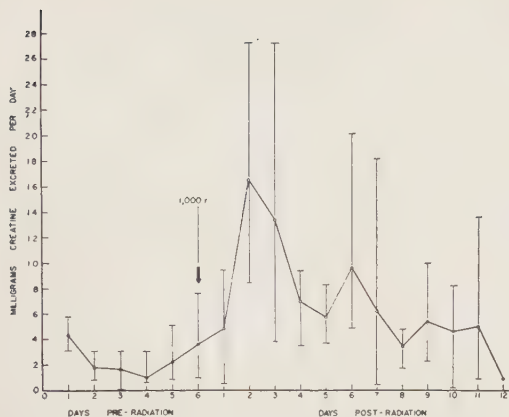


FIG. 1. Daily urinary creatine excretion of 4 male adult monkeys before and after 1,000 rep of X-irradiation. Each circle represents the mean and the barred lines represent the extremes excreted.

cessive amounts of creatine in the urine immediately following irradiation(2). In this paper we wish to report evidence for post-irradiation creatinuria in the monkey.

Materials and methods. Four normal adult male *Macaca mulatta* monkeys approximately 3 to 4 years old, weighing an average of 10 lb, were placed in metabolism cages and fed a creatine-free diet(1). After stabilization on the diet for 7 to 9 days, the animals were anesthetized with 10 mg/lb of Nembutal and were irradiated with a Picker X-ray machine at the rate of 75 r/min. for a total dose of 1,000 rep. Constants of the machine were 250 KVP, 18 Ma, filter 1.0 mm Al plus 0.25 mm Cu at a distance of 60 cm from target to object. Dosimetry in air was performed with a Victoreen rate-meter which had been previously calibrated against a source from the National Bureau of Standards. Urinary creatine determinations were made by the method of Anderson *et al.*(3).

Results. Two of the animals excreted large amounts (19.7 and 26.2 mg, respectively) of creatine on the second day of the diet. By the third day of the diet, however, all animals had become stabilized to a consistently low level of urinary creatine excretion. The average daily excretion for the 4 animals for the 6 days prior to irradiation was 2.5 mg. The average excretion was approximately twice normal on the first postirradiation day, 7 times normal on the second postirradiation

day, and 5 times normal on the third post-irradiation day (Fig. 1). A secondary creatinuria occurred on the sixth day when the average was approximately 4 times normal. One animal died on the eighth, 2 on the twelfth, and one on the thirteenth post-irradiation day.

Discussion. The results in these 4 monkeys are clear: a postirradiation creatinuria about 7 times normal occurred in all animals. This picture is very similar to that found in the rat(1). The terminal creatinuria which we have observed in most of our rats occurred in one monkey (15.8 mg on the last day of life), but not in the other 3.

Zhahova and Broun(4) state that postirradiation creatinuria was first observed in man in 1938 and was subsequently confirmed by experiments on dogs. Unfortunately, no references are given for these statements, nor are any of the details as to dose, magnitude of effect, etc., discussed. These authors do, however, publish figures for postirradiation creatinuria in 90 rats. An approximate 4- to 5-fold creatinuria on the second day followed an acute dose of 500 rep of X-rays. Haberland *et al.*(5) report an approximate 2-fold creatinuria on the second postirradiation day following 759 r of X-ray in rats. However, the control values for daily creatine excretion are higher than any we have ever encountered, and since these animals were not fed a creatine-free diet prior to radiation, it is possible that the experiments are not entirely comparable to our own. The discrepancy between our results for the dog(2) and those reported by Zhahova and Broun may be due to a difference in dose administered. Some of the details of previously published work are given in Table I.

Summary. Four adult male *Macaca mulatta* monkeys weighing an average of about 10 lb were placed on a creatine-free diet for 7 to 9 days. After the urinary level of creatine excretion had reached a plateau, the animals were irradiated with 1,000 rep of X-rays. A creatinuria of approximately 4 times normal was excreted over the first 4 days in a pattern similar to that previously found in the rat. On the second postirradiation day, the

TABLE I. Postirradiation Creatinuria in Various Species.

Species	Year	No. of cases	Type of radiation	Dose of radiation	Creatinuria	Method used	References
Man	1938	1	Not given	Not given	+	Not given	Quoted, (4)
Dog	1956	Not given	" "	" "	+	" "	" (4)
Dog	1957	2	X-ray	400-500 r	—	Diacetyl test	(2)
Rat	1955	19	"	759 r	+	Method of Stelgen	(5)
Rat	1956	90	"	500 r	+	Not given	(4)
Rat	1957	61	Neutrons	435 rep	+	Diacetyl test	(1)
			Co ⁶⁰ gamma	750-1500 rep	+	" "	
			X-ray	1,500 r	+	" "	
Monkey	1957	4	X-ray	1,000 r	+	Diacetyl test	This report

average creatinuria was about 7 times normal. This effect is compared with other species reported in the literature.

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Received June 26, 1957. P.S.E.B.M., 1957, v95.

Biotin Requirements of a Mutant Strain of *Escherichia coli** (23358)

ROBERT B. FERGUSON AND HERMAN C. LICHSTEIN

Department of Bacteriology and Immunology, University of Minnesota, Minneapolis

Biotin, in common with other members of the B vitamin group, is required or synthesized by all microbial cells. Normally, strains of *Escherichia coli* are capable of growth in the absence of added biotin since they are able to synthesize this vitamin. The strain of *E. coli* employed in the present studies was obtained[†] by the treatment of a normal wild type with a mutagenic agent and represents a biotinless mutant of *E. coli*, hence a strain which has lost the ability to synthesize biotin.

The present paper provides a study of the biotin requirements of this biotinless mutant strain of *E. coli* including factors both nutritional and environmental which alter these requirements.

Materials and methods. The biotin-require-

ing mutant strain of *E. coli* was designated M8178.[†] The basal medium contained 1 g each of KH₂PO₄, K₂HPO₄ and NaCl, 0.7 g of MgSO₄ · 7H₂O, 0.5 g of sodium citrate, 4 g of (NH₄)₂SO₄ and 5 g of vitamin-free caseamino acids (Difco) per 800 ml of distilled water. The pH prior to sterilization in the autoclave (121°C, 15-20 min.) was 6.8. The commercial vitamin-free caseamino acids were purified further by charcoal adsorption to remove traces of biotin(1).

The inoculum was prepared by subculturing the organism in 5 ml of inoculum medium (1% each of yeast extract, casitone and glucose, and 0.5% of K₂HPO₄). After incubation at 30°C for 12-18 hrs, the cells were harvested by centrifugation and washed 3 times in 5 ml of saline. The assay vessels employed for stationary cultivation were 16 x 150 mm tubes covered with aluminum caps. Suitable concentrations of biotin or other materials were added to the tubes in aliquots of 1 ml or

* Supported in part by a grant from the National Vitamin Foundation; and by the Graduate School Research Fund, University of Minnesota.

[†] Kindly furnished by Dr. B. D. Davis of New York Univ. Med. School.

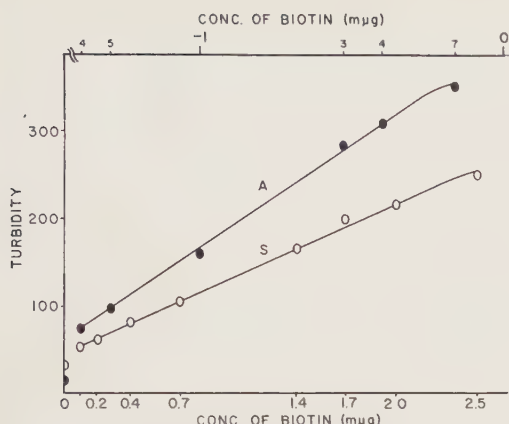


FIG. 1. Response of *E. coli* to biotin. 36 hr incubation at 30°C. S = Stationary incubation (arithmetic scale). A = Aerobic incubation (logarithmic scale).

less and the volumes adjusted to 1 ml with distilled water. The tubes were sterilized by autoclaving for 15 min. at 121°C. After cooling, 4 ml of previously inoculated medium (1 drop of inoculum diluted 1:10 per 100 ml of sterile medium) were added to each tube. The contents were mixed by shaking and incubated at 30°C for 30-36 hrs. For aerobic cultivation the assay vessels were 25 x 150 mm tubes fitted with one hole rubber stoppers plugged with cotton, and incubation was carried out on a reciprocal shaking machine.

Growth response was determined turbidimetrically with a Klett-Summerson photoelectric colorimeter equipped with a blue filter (400-450 mμ). Prior to reading, each tube was shaken vigorously to suspend the cells and the instrument set at zero with a blank containing 4 ml of uninoculated medium and 1 ml of water.

Results. Typical growth responses of this organism to varying concentrations of biotin under stationary and aerobic conditions of incubation are given in Fig. 1. It is to be noted that the dose-response curve of this organism to biotin appears to depend on degree of aeration during cultivation. Thus, a linear turbidity increase with arithmetic increases in biotin concentration was obtained under stationary conditions of cultivation, while a straight line response was obtained under aerobic conditions of incubation only when the log concentration of the vitamin was em-

ployed. The linear response to logarithmic increases in concentration of biotin is typical of most microorganisms employed for assay of this vitamin (*i.e.*, species of *Saccharomyces* and *Lactobacillus*), whereas the straight line response to arithmetic concentrations of biotin has been reported previously only for propionibacteria(2). For assay purposes, the usable range of response extends from 0.1-2.5 μg of biotin for stationary cultures and from 0.04-0.7 μg for aerated cultures. Linearity was maintained below the indicated lower limits, however in this range relatively large changes in vitamin concentration produced small differences in turbidity.

The next phase of the study was an investigation of the influence of certain nutritional factors on response of this strain of *E. coli* to biotin. For this study stationary conditions of incubation were employed.

Inasmuch as biotin has been implicated in certain areas of nitrogen metabolism, it appeared desirable to inspect the effect of concentration of amino acids in the growth medium on the response of *E. coli* to biotin. It was found that 0.5% casamino acids in the medium was optimal for the response of this microorganism to biotin considering range of linear response, sensitivity and reproducibility of results. At low concentrations of casamino acids (0.1%) growth was limited and range of response to biotin was quite narrow as might well be expected since the amino acids serve as the principal source of energy in the absence of added carbohydrate. High concentrations of casamino acids (1-2%) resulted in marked inhibition of growth at low levels of biotin, and this inhibition was overcome by the presence of optimal amounts of this vitamin. It is possible that growth inhibition may have resulted from the inefficient metabolism of some amino acids due to a deficiency in biotin-linked reactions with an accumulation of such amino acids in toxic amounts. The adverse effect of amino acids on bacteria under certain conditions is well recognized and was reported first by Gladstone(3) working with *Bacillus anthracis*.

The effect of inclusion of glucose in the medium on biotin requirements of *E. coli* is

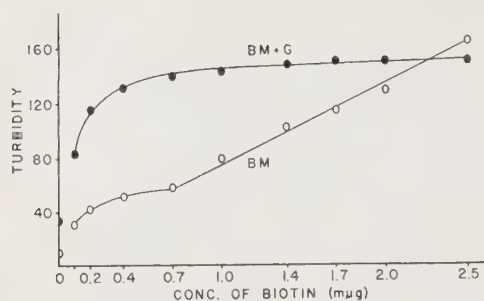


FIG. 2. Effect of inclusion of glucose in growth medium on response of *E. coli* to biotin. 36 hr incubation at 30°C under stationary conditions. BM = Basal medium. BM + G = Basal medium + 1% glucose.

shown in Fig. 2. The most striking effect was the marked sparing action of glucose on the biotin requirements of this organism. This sparing effect was noted not only at sub-optimal levels of biotin but was reflected also in the absence of added biotin (*i.e.*, the tubes containing basal medium plus glucose but without added biotin routinely supported 3-5 times as much growth as identical tubes without glucose). This effect of glucose was quite surprising and the mechanism is still under investigation. The increased growth in the presence of glucose does not appear to be related to biotin synthesis since microbiological assays of acid hydrolyzed cells with *Saccharomyces cerevisiae* (139) as the assay organism revealed the same biotin content for cells harvested from media without added biotin in the presence or absence of glucose. Furthermore, avidin did not antagonize this effect of glucose when incorporated into the growth medium.

The possible use of this biotinless mutant strain of *E. coli* for microbiological assay of biotin would appear to be limited to samples contributing amino acids and/or glucose in amounts less than that required to produce the disturbing effects discussed above. It is probable therefore that use of this organism as an assay tool will be limited to research problems with relatively pure samples.

It is well established that incorporation of certain fatty acids, particularly the 18-carbon unsaturated members, reduces the requirement for biotin by lactic acid bacteria(4). It was desirable therefore to ascertain the ef-

TABLE I. Effect of Tweens on Response of *E. coli* to Biotin.

Additions	Turbidity				
	None	Biotin (mμg/tube)			
		.1	1.0	3.0	5.0
None	2*	25	106	199	224
Tween 80, 10 μg	4	38	153	234	226
" " , 100	3	32	57	68	73
Tween 20, 10	2	37	158	238	222
" " , 100	3	38	58	72	79

* Klett-Summerson readings after 36 hr incubation at 30°C.

fect, if any, of fatty acids on response of *E. coli* to biotin. This was determined by using oleic and lauric acids in the form of Tween 80 and 20 respectively (Table I). The results reveal that under stationary conditions of incubation both Tween 20 and 80 at a concentration of 10 μg/tube caused a stimulation of growth at sub-optimal levels of biotin. No increase in total growth was noted and non-specificity was suggested by the fact that both Tweens worked equally well. At higher concentrations (100 μg/tube) definite inhibition of growth was noted, which is in keeping with observations with other microorganisms. It is pertinent that the Tweens had no effect on growth of this organism in the complete absence of biotin which agrees with the recent work of Traub and Lichstein(5).

The data presented in Table II reveal the stimulatory effect of CO₂ on growth of *E. coli* incubated under stationary conditions in the presence of biotin. Although the interpretation of these results is of necessity limited, it

TABLE II. Effect of CO₂ on Response of *E. coli* to Biotin.

Biotin (mμg/tube)	Turbidity	
	Air	CO ₂ *
0	10†	16
.2	42	44
.4	50	60
.7	56	81
1.0	87	134
2.0	126	201
2.5	164	210
5.0	223	270

* Incubated in closed container with enough solid carbon dioxide (dry ice) to yield 1 atmosphere of gas.

† Klett-Summerson readings after 36 hr incubation at 30°C.

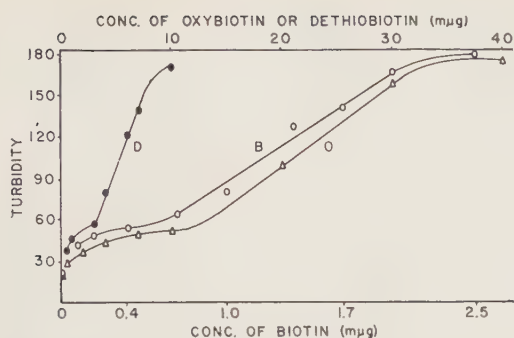


FIG. 3. Response of *E. coli* to biotin, oxybiotin and dethiobiotin. 22 hr incubation at 30°C under stationary conditions. B = D-biotin. O = DL-oxybiotin (O—heterobiotin). D = DL-dethiobiotin.

would appear that CO_2 is an essential substance in the metabolism of *E. coli* and that biotin is concerned with production of metabolic CO_2 . The function of biotin in the decarboxylation of oxalacetate and succinate is known(6), and the sparing effect of CO_2 on biotin requirements of other organisms has been reported(5). The fact that the CO_2 effect was greater in the presence of increased amounts of biotin may reflect the utilization of CO_2 in biotin-linked CO_2 fixing systems resulting in formation of substances necessary for optimal growth of this organism.

Microorganisms vary widely in their ability to utilize analogues of biotin. This likely depends both upon specificity and ability to

convert the analogue into a usable form. The comparative results obtained with biotin, oxybiotin[†] and dethiobiotin with *E. coli* under stationary conditions of incubation are shown in Fig. 3. It is manifest that both analogues were utilized by *E. coli* although not as effectively as biotin. If one assumes that only the D-isomer is active biologically then the values given for oxybiotin and dethiobiotin should be halved. The reduced activity of both analogues (and especially oxybiotin) suggests either that they are not efficiently converted to biotin by this organism or that they are used metabolically in a form different from that produced from biotin.

Summary. The biotin requirements of a biotinless mutant strain of *E. coli* are presented. Data on the influence of degree of aeration and changes in nutrition on the response of this organism to biotin are included.

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[†] We are indebted to Hoffmann-LaRoche, Inc. for a generous supply of this compound.

Received June 26, 1957. P.S.E.B.M., 1957, v95.

Influence of Dietary Sodium Chloride on Incidence of Urinary Calculi in Sheep.* (23359)

C. J. ELAM, W. E. HAM, AND B. H. SCHNEIDER
(Introduced by James McGinnis)

Department of Animal Science, State College of Washington, Pullman

Addition of potassium acid phosphate (K_2HPO_4) to diets fed to sheep has been reported to greatly increase the incidence of

urinary calculi(1). Further studies(2) have indicated that both phosphorus and potassium are important in the etiology of urinary calculi in sheep. The largest occurrence of cal-

* Scientific Paper no. 1524. Washington Agri. Exp. Station, Pullman. Project No. 1001. Presented by C. J. Elam as part of thesis in partial fulfillment of requirements for Ph.D. degree.

This investigation was supported in part by funds provided for biological and medical research by State of Washington Initiative Measure No. 171.

TABLE I. Composition of Diets, Lb.

	1	2	3	4	5	6	7	8	9	10	11	12
Wheat straw	44.7	40.5	44.7	40.5	43.8	39.7	43.8	39.7	19.7	18	19.7	18
Sugar	5	4.5	5	4.5	5	4.5	5	4.5				
Ash of beet pulp					.9	.8	.9	.8				
Beet pulp (with- out molasses)									30	27	30	27
Wheat	15	13.5	15	13.5	15	13.5	15	13.5	15	13.5	15	13.5
Oats	17	15.2	17	15.2	17	15.2	17	15.2	17	15.2	17	15.2
Linseed meal	17.8	16.3	17.8	16.3	17.8	16.3	17.8	16.3	17.8	16.3	17.8	16.3
NaCl	.5	10	.5	10	.5	10	.5	10	.5	10	.5	10
K ₂ HPO ₄			2.4	2.4			2.4	2.4			2.4	2.4
Vit. A and D*												
Calcium, %	.291	.231	.297	.291	.378	.638	.421	.456	.491	.387	.447	.387
Phosphorus, %	.249	.297	.496	.626	.383	.388	.728	.708	.360	.282	.708	.738
Magnesium, %	.153	.133	.143	.128	.259	.178	.120	.184	.240	.215	.115	.208
Potassium, %	.884	.758	1.373	1.474	.751	.671	1.775	1.638	.669	.579	1.654	1.666
Sodium, %	.338	3.350	.325	4.000	.407	2.960	.310	3.920	.362	3.500	.369	2.830
Chloride, %	.843	6.030	.728	6.381	1.062	5.320	.442	5.720	.667	5.939	.843	5.320

* 325 I. U. vit. A and 100 I. U. vit. D/lb of feed.

culi resulted in sheep supplemented with both phosphorus and potassium rather than either one singly. Lindley *et al.*(1) reported that 25% of molasses dried beet pulp in diets fed to sheep increased the incidence of urinary calculi. Later investigations by Elam *et al.* (2) indicated beet pulp rather than molasses in molasses dried beet pulp was responsible for the predisposing effect on urinary calculi formation. However, the factor(s) in beet pulp responsible for this effect is not known. Since surgery is often necessary in the treatment of animals afflicted with the disease, the development of a preventive treatment is desired. That a large intake of sodium chloride causes diuresis in animals is well known. Meyer *et al.*(3) reported that feeding sodium chloride to 9.4% of the diet did not adversely affect the average daily gains or efficiency of feed utilization of fattening sheep and cattle. Further, there is evidence that an interrelationship exists in the body between sodium and potassium(4).

The objectives of this experiment were: (1) to obtain additional information on the factor in beet pulp responsible for its predisposing effect on calculi formation, to observe the effects of adding potassium acid phosphate to the diet, and (2) to study the influence of adding a relatively high level of sodium chloride to the diet on the incidence of urinary calculi.

Procedure. Sixty crossbred wether lambs, averaging 84 lb, were divided into 12 lots of

5 lambs each and group-fed a pelleted diet twice daily. The diets were supplemented with adequate levels of vit. A and D to eliminate the possible influence of a low level of these vitamins on the formation of calculi. Water was available *ad libitum*. Variables in the diets were: potassium acid phosphate (K₂HPO₄), sodium chloride, beet pulp (without molasses), and ash of beet pulp. These variables were included in a factorial design (Table I). Beet pulp ash was fed to determine if the causative factor in beet pulp is inorganic. Beet pulp was supplied at approximately 30% of the diet in 4 of the 12 lots. The ash was included in another 4 diets at a level of 0.8 to 0.9%, equivalent to the amount of ash supplied by the beet pulp added. Ash was obtained by first collecting the ash of beet pulp burned in 50-gallon drums. This ash was then placed in a muffle furnace for 15 minutes at 600°C. In the remaining lots neither beet pulp nor its ash was supplied. Sodium chloride was included at 0.5% in half of the diets and at 10% in the other half. The 10% level of salt was arbitrarily selected to furnish a large salt intake, without restricting feed consumption. Potassium acid phosphate was added to half of the diets at 11 g per lb of diet, the level reported to increase the occurrence of urinary calculi(1,2). Lambs were observed daily to detect symptoms. When noticed, the lamb was sacrificed and autopsied. At conclusion of the experiment, the remaining animals were

TABLE II. Incidence of Urinary Calculi by Treatment.*

NaCl, %	No beet pulp		Ash of beet pulp		Beet pulp		Total
	Control	K ₂ HPO ₄	Control	K ₂ HPO ₄	Control	K ₂ HPO ₄	
10	0	0	0	0	0	0	0
.5	0	4	1	4	2	3	14

* 5 sheep on each treatment.

slaughtered and the entire urinary tract of each was removed and examined for calculi. Those having stones present at slaughter were included as calculi cases along with the lambs already autopsied. Calculi recovered from the urinary tracts were not chemically analyzed in this experiment. Calculi in sheep receiving similar rations as fed in this experiment were reported to contain principally calcium and magnesium phosphate(1,2).

Results. During the 105 days of the observation, 10 animals displayed symptoms of urinary calculi, with the first occurring on 33rd day. The symptoms observed were anorexia, restlessness, and straining in an attempt to urinate. The afflicted lambs often stood with their hind feet placed far to the rear and frequently shifted their weight on their rear legs. At the time of autopsy, the urinary tract was usually damaged extensively. The bladder was distended severely and kidneys were often in a state of acute hydronephrosis. Calculi were found in the bladder in almost every instance, but in only 2 cases were stones found in the kidneys. The site of occlusion of the urinary tract with calculi was usually the urethral process.

At the conclusion of the experiment, when the remaining 50 lambs were slaughtered, 4 more lambs had calculi in their urinary tracts. Thus, a total of 14 cases of calculi developed during the experiment (Table II).

Addition of 10% sodium chloride to the diets of 30 sheep prevented occurrence of urinary calculi. No decrease in feed consumption was observed in the lambs receiving this high level of salt. However, they did void considerably more urine as evidenced by the condition of the litter. The 14 cases of calculi that resulted were in the 30 sheep receiving 0.5% of added sodium chloride in their diets.

Of the sheep which received the lower level

of sodium chloride in their ration, 15 also received the potassium acid phosphate supplement. Eleven of these animals developed calculi, while 3 of the 15 not receiving potassium acid phosphate formed calculi. Thus, 11 of the 14 cases of urinary calculi occurred in sheep fed the 0.5% level of sodium chloride plus potassium acid phosphate. This follows closely the results of previous studies in which potassium acid phosphate caused an increased incidence of calculi.

The addition of the ash of beet pulp or of beet pulp itself to the diet did not alter the incidence of calculi. There were 5 cases produced, both when the ash was added and when beet pulp itself was added. Four cases occurred in the lots receiving neither of the supplements. These results do not agree with those of previous studies in which beet pulp increased the incidence of calculi. The reason for this lack of agreement is unknown. Apparently the beet pulp varied enough in composition from that used in the previous experiments to cause this difference in results. It is interesting to note that the 3 sheep that developed calculi in the absence of added potassium acid phosphate were receiving either beet pulp or its ash.

Discussion. The reason for the prophylactic effect of the high level of sodium chloride in the diet is not known, but there are several possible explanations. Perhaps the diuresis resulting from feeding a diet high in salt is sufficient to wash any stones which may form out of the urinary tract before their size is great enough to block urine passage. On the other hand, the prophylaxis may be due to inhibition of calculi formation. The increased volume of urine voided may dilute the crystalloids present to such an extent that precipitation is prevented, or at least hindered.

Since sheep urine is normally alkaline in reaction the preventive effect may have been

mediated through a decrease in urine alkalinity. Investigations with rats(5) and mink (6) have indicated that the addition of ammonium chloride to the diet prevented urinary calculi formation. Leoschke and Elvehjem (6) reported that the addition of one gram of ammonium chloride daily to a diet fed to mink reduced the incidence of urinary calculi. They attributed the effect to the increase in urinary acidity. Feeding sheep a 10% level of sodium chloride in diets similar to those fed in this experiment has resulted in an increase in urinary acidity from an average pH of 7.95 to 7.30.[†] Using analysis of variance, this difference was statistically significant ($P < .01$). The urine pH was determined by collecting a 24-hour urine sample under toluene and immediately measuring the pH of the urine with a glass electrode pH meter.

The effect of the high salt diet may be related to the relationship which exists in the body between sodium, potassium and other minerals. Of course, a combination of several or all of the above mentioned factors may have been responsible for the preventive effect of the 10% level of sodium chloride. Whatever the reason, it should be emphasized that additional investigation is needed before the use of a high sodium chloride diet to prevent urinary calculi can be recommended.

[†] Elam, C., and Ham, W., unpublished data.

Summary. Sixty wether lambs were divided into 12 lots of 5 each and fed the experimental diets for a 105-day period. The animals were group fed all they would consume twice daily. The variables included in the diets were the presence and absence of potassium acid phosphate (K_2HPO_4), a 10% level of sodium chloride, a 30% level of beet pulp, and the ash of the same amount of beet pulp. Fourteen cases of urinary calculi resulted in the 60 sheep. None of these cases occurred in the 30 sheep which received the 10% level of sodium chloride. Eleven of the 14 cases of calculi occurred in those lots receiving added potassium acid phosphate in the diet, while 3 occurred in its absence. Beet pulp or beet pulp ash had no effect on the incidence of urinary calculi.

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Received December 6, 1956. P.S.E.B.M., 1957, v95.

Effect of Vitamin D on Retention of Radium by the White Rat.* (23360)

ROBERT C. HILTIBRAN,[†] F. E. HOECKER, AND R. C. MILLS
(Introduced by H. W. Barrett)

Departments of Biochemistry and Physics, University of Kansas, Lawrence

The effect of vit. D on retention of radium in white rats was studied as part of a general investigation on distribution and behavior of radium in the animal body. It has been believed since the early studies on radium poisoning that radium is deposited and retained

in the skeletal structure. Evans *et al.* (1), using radioautographs, confirmed this belief, showing that radium was concentrated in the region of the epiphyses. Norris and Kisieleski (2) found that 3 days after intravenous administration of radium skeletal retention was 63% of the injected dose; retention was still 53% after 30 days. Since total body retention after the 30 days was 55% of the injected dose, practically all the radium re-

* Supported in part by contract between University of Kansas and Office of Naval Research.

[†] Present address: Dept. of Agric. Biochem., Ohio State University, Columbus.

tained was in the skeleton. Qualitatively similar results were obtained with Ca^{45} and $\text{Sr}^{89,90}$. Greenberg (3) found that the amounts of Ca^{45} and Sr^{89} retained in the skeletons of rachitic rats 3 days after intraperitoneal injection of the radioisotopes were markedly increased by administration of irradiated ergosterol to the rats. Retention in the soft tissues was insignificant. In addition, vit. D increased absorption of oral doses of the isotopes. Skeletal uptakes of injected (intravenous) Ca^{45} and $\text{Sr}^{89,90}$ were shown by Copp and coworkers (4) to be very rapid in young rats, reaching a maximum within an hour. The deposited isotopes were removed only slowly from the bones. Young rachitic (low P) rats showed the same rapid initial uptake as normal animals, but much more rapid loss occurred. Skeletal retention after 2 to 3 days was therefore much lower than in the normal rats, and after 8 days was less than one-third that in normal animals. These workers also found that skeletal retention of Ba^{140} in the low P rachitic rats was only half that in normal rats after 8 days. Dudley and Friedman (5) found that retention of Ga^{72} in the ends of the long bones of rachitic rats was increased 40% by administration of vit. D, which had no effect on deposition in the shafts. Considerable Ga^{72} was readily deposited in the bones of rachitic rats even when no concurrent deposition of calcium salts occurred.

The experiment reported here was designed to study the effect of low P rickets, and vit. D treatment thereof, on radium retention in the rat. Unpublished results of preliminary experiments indicated that rachitic rats on a vit. D-free, high-calcium, low-phosphorus diet retained less radium 30 days after a radium injection than did vit. D-treated rachitic or normal control animals. Similar results of a more extended investigation are presented here.

Procedures. Male rats of the Sprague-Dawley strain, 21 days old, average weight about 40 g, were divided by random selection into 3 groups: (A) control group (14 rats); (B) untreated-rachitic group (20 rats); (C) vit. D-treated rachitic group (13 rats). The animals were quartered in individual cages

in a constant temperature room, with free access to water and U.S.P. Rachitogenic Diet No. 2. The animals of the control group were fed, by medicine dropper, 40 International Units of Vitamin D per day as a solution of calciferol in corn oil. The animals of the treated-rachitic group, after 40 days on the rachitogenic diet, were given 80 I.U. of vit. D per day for a period of 4 days prior to injection of radium, then received 40 I.U. per day until sacrificed. All animals not receiving vit. D were given the corresponding amount of corn oil daily. Forty-four days after the animals had been placed on the rachitogenic diet each rat received an intraperitoneal injection of 1 ml of physiological saline solution containing 10.6 μg of radium, as radium chloride. (Radium contents of several samples were estimated by gamma radiation determinations.) The animals were sacrificed by anesthesia with ethyl ether 23 days following injection of the radium, then each carcass was hermetically sealed in a thin-walled metal can, and placed in a deep-freeze. The radium content of each can was estimated after a 30 day period by measurement of gamma radiation with a Geiger counter, and comparison with a radium standard. Five animals from the control group and 6 animals from the untreated-rachitic group were sacrificed at time of injection of radium into the remaining animals of the experiment, and bone ash was determined on dry, fat-free long bones of the hind legs of each animal. The bones were ashed at 750°C in an electric muffle furnace. Per cent of bone ash was calculated as the weight of remaining ash $\times 100$, divided by the weight of the fat-free, moisture-free bone.

Results. The data (Table I) show that the animals of the untreated-rachitic group retained significantly less radium than did the animals of either the vit. D-treated rachitic group or the control group. Greatest individual variations in amount of radium retained were observed in the untreated-rachitic group, where retention ranged from 1.97 to 3.86 μg , while control and vit. D-treated groups did not exhibit such wide variations.

That the untreated animals were rachitic at time of injection of radium is shown by

TABLE I. Radium Retention in Rachitic and Vit. D-Treated Rats.

Group	No. of rats	Bone ash, %	Mean radium retention, μg
I. Normal control	9		$3.98 \pm .128^*$ (3.61-4.77)
	5†	60.78 (60.38-61.35)	
II. Untreated rachitic	14		$2.90 \pm .141^*$ (1.97-3.65)
	5†	52.93 (45.99-57.40)	
III. Vit. D-treated rachitic	13		$4.36 \pm .063^*$ (4.02-4.76)

* Stand. error.

† Killed at same time that radium was inj. into other animals of group.

the bone ash determinations on animals from the rachitic and control groups, sacrificed at time of injection of radium into the rest of the animals of the groups. The rachitic animals showed significantly lower bone ash levels than did the control animals (Table I). Variation between bone ash values of the rachitic group was much greater than in the control group.

The high variation of bone ash levels of the rachitic animals corresponds to the variation in radium retention in this group, in contrast to the relatively small variations in the control and treated groups.

Discussion. Significantly more radium was retained by the animals receiving vit. D than by the untreated rachitic animals. Differences in mean radium retention between Groups I and II, and between Groups II and III, are highly significant, *t* values being 5.33 and 9.19 respectively. Even the difference between Groups I and III has a probability

of only 1%, so is also significant. It was assumed, because of the previous studies by many other workers, that total body retention of radium 23 days after administration of the radium would be analogous to skeletal retention. The results of our experiments do not permit us to decide whether the effect of vit. D is on the initial deposition of radium in bones, or on rate of removal from the bones.

It would appear that retention of radium, therefore, is affected by the absence or presence of vit. D in the diet in the same manner as are the retentions of Ca^{45} , $\text{Sr}^{89,90}$, and Ba^{140} . Since it is impossible to demonstrate in rats a requirement for vit. D on diets with normal Ca and P contents, the high Ca-low P diet was used to demonstrate these effects on radium retention. There is of course no assurance that vit. D would affect Ra retention in rats on normal diets, or in humans.

Summary. Total body retention of injected radium was significantly less in rachitic rats than in vit. D-treated rachitic rats, or in non-rachitic control animals.

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Received January 21, 1957. P.S.E.B.M., 1957, v95.

Hypothalamic Lesions Associated with Goldthiogluucose-Induced Obesity.* (23361)

ROBERT A. LIEBELT AND JOHN H. PERRY (Introduced by Arthur Kirschbaum)

Department of Anatomy, Baylor University College of Medicine, Houston, Texas.

A massive and persistent obesity can be induced in various strains of mice with a single

* This investigation supported by Public Health Service Grants.

injection of goldthiogluucose(1-4). Of those animals surviving the LD₅₀ dose about one-third show varying degrees of fat deposition (5). This obesity is associated with a marked

increase in spontaneous food intake; carcass analysis indicates that increase in body weight is essentially due to increase in body lipid (6).

Marshall, Barnett and Mayer (7) described lesions in the ventromedial nuclei of the hypothalamus of animals injected with goldthioglucose 24 to 72 hours prior to autopsy. Neurons were scarce in the ventromedial nuclei of mice made obese by injection of gold thioglucose at least 3 months prior to investigation. Sections of brains of mice which failed to become obese after injection with goldthioglucose at least 3 months prior to autopsy revealed no distinct abnormalities. No lesions were observed in other areas of the brain.

Our observations indicate that 2 genetically unrelated inbred strains of mice (CBA and C₅₇BL) demonstrate differences in susceptibility to goldthioglucose-induced obesity. One hundred percent of CBA mice become obese with no mortality following a given dose of goldthioglucose which has no effect on weight gain of C₅₇BL mice. However, larger doses of goldthioglucose, which are fatal for CBA mice, produce significant weight gain in C₅₇BL mice. It was the purpose of this investigation to determine the relationship between weight gain following goldthioglucose and the nature of the lesions in the hypothalamus in these 2 strains of mice.

Methods. The strains of mice used had been inbred in this laboratory for more than 20 generations; mice were 3 months of age when goldthioglucose was administered. Purina lab chow was fed *ad libitum*; mice were housed in groups of 4-6 animals per cage (6 x 12 inches). The goldthioglucose suspension was prepared by adding 100 mg to 2 cc of peanut oil which was vigorously agitated prior to each injection. (Our recent studies show that goldthioglucose dissolved in saline is as effective in producing obesity.) Most injections were made intraperitoneally; previous experience indicated no difference between the intramuscular and intraperitoneal routes. A total of 100 CBA mice were injected with 0.18 to 0.75 mg[†] of goldthioglucose, compared

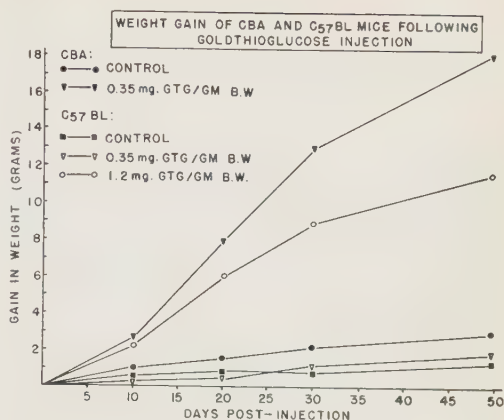


FIG. 1. Response in terms of wt gain to varying doses of goldthioglucose in CBA and C₅₇BL inbred mice. Each group represents 15-25 mice.

to the LD₅₀ dose of 0.80 to 1 mg per gram used by others (1-4); a total of 50 C₅₇BL mice were injected with 0.18 to 1.2 mg of goldthioglucose. Body weights were taken at weekly intervals for a period of 50 days post-injection. The response in terms of weight gain to different amounts of goldthioglucose was consistently reproducible in both strains of mice. Another group of mice was killed at intervals varying from 2-19 days post-injection. Stained serial sections of the brains of 23 CBA and 19 C₅₇BL mice were studied. Twenty CBA and 16 C₅₇BL had received goldthioglucose and 3 animals from each strain served as controls. The usual amount of drug was 0.35 mg, but a few animals received smaller (0.18 mg) or larger doses (0.75 to 1.2 mg).

Results. The average weight gain of CBA mice injected with 0.35 mg of goldthioglucose was 19 g with no mortality at 50 days post-injection (Fig. 1). The control animals during this time gained 2 g. Control C₅₇BL mice as well as those animals injected with 0.35 mg of goldthioglucose showed a 1 to 1.5 g gain in weight at 50 days post-injection. However, animals of this strain receiving 1.2 mg of goldthioglucose gained an average of 11 grams during this same period. Both strains of mice had approximately the same body weight (23-25 g) prior to injection.

Although in C₅₇BL mice 0.35 mg of goldthioglucose caused no significant weight increase, bilateral lesions (Fig. 2) appeared in

[†] Dosage of goldthioglucose is expressed in mg/g body weight.

PLATE I. Photomicrographs of sections through infundibular region of hypothalamus of goldthioglucoase treated mice. ME, median eminence; VM, nucleus ventromedialis; A, nucleus arcuatus; III, third ventricle; L, lesion.

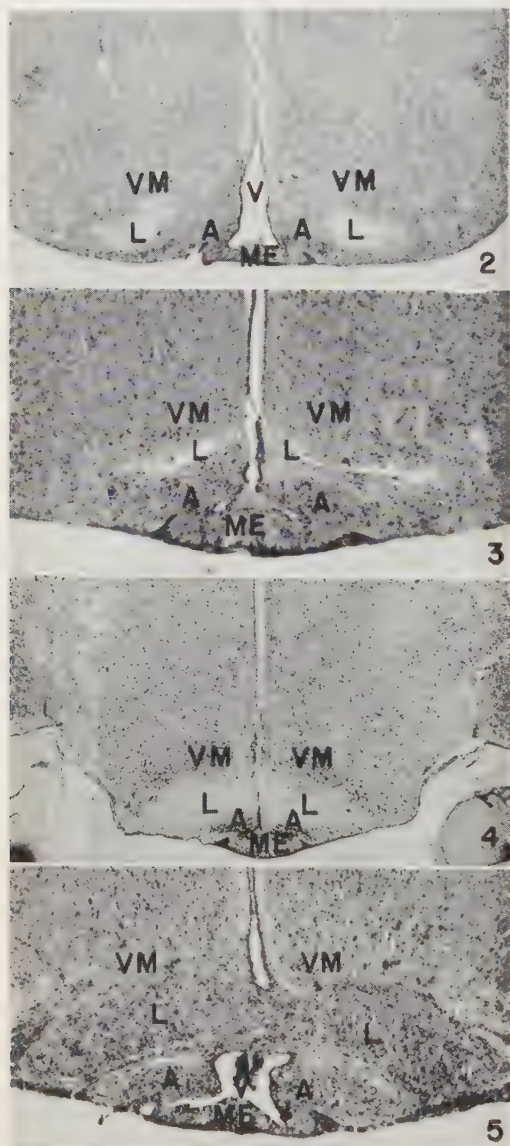


FIG. 2. Bilateral hypothalamic lesions *unassociated* with wt gain in $C_{57}BL$. Dose: .35 mg/g body wt. Duration of experiment: 2 days. Stain: Romanes Silver. $\times 23$.

FIG. 3. Bilateral hypothalamic lesions *unassociated* with wt gain in CBA. Dose: .18 mg/g body wt. Duration of experiment: 19 days. Stain: Hematoxylin and eosin. $\times 23$.

FIG. 4. Hypothalamic lesion associated with marked wt gain in CBA. Dose: .35 mg/g body wt.

Duration of experiment: 2 days. Stain: Toluidin Blue. $\times 45$.

FIG. 5. Hypothalamic lesion associated with moderate wt gain in $C_{57}BL$. Dose: 1.2 mg/g body wt. Duration of experiment: 19 days. Stain: Hematoxylin and eosin. $\times 45$.

the hypothalamus. These extended from the mammillary bodies to the optic chiasma and were localized in the cell-poor interval between the ventromedial nuclear complex and the arcuate (infundibular) nucleus. *Similar lesions* (Fig. 3) occurred in CBA mice with a dose of 0.18 mg. CBA mice receiving this low dose showed no significant weight gain.

On the other hand, doses of 0.35 mg in CBA mice produced large lesions (Fig. 4), which were correlated with a great increase in body weight. In the $C_{57}BL$ strain lesions of comparable magnitude (Fig. 5) resulted only when the dose of goldthioglucoase was increased 3 to 4 times (1.2 mg). As indicated above, weight gain in the $C_{57}BL$ strain was not as great as in the CBA, although the hypothalamic damage was similar (Fig. 4,5). CBA mice which receive more than 0.75 mg of goldthioglucoase usually die within 3 to 4 days post-injection, and autopsy reveals gastric ulcers and stomachs overdistended with food. The brains of these animals show destruction of all of the nuclei of the hypothalamus except the supraoptic, dorsal parts of the filiform group, and the most ventral cells of the arcuate nuclei.

Lesions associated with significant increase in body weight differed from those unattended by weight increment, in that there occurred considerable destruction of the arcuate nucleus and ependymal cells of the third ventricle in the former (Fig. 4,5). Differences in rostral and caudal limits of the lesions in the obese and non-obese animals were not great.

All CBA mice with significant increase in body weight showed lesions in the limbic system (Fornix, ventral psalterium, and primordial hippocampus), (Fig. 7,8), in addition to hypothalamic damage. The histopathologic features of limbic and hypothalamic lesions were similar. Alteration of background staining, edema, and hemorrhage predominated in cases with 2- to 3-day experimental intervals. Macrophages were abundant in 5- and 9-day

PLATE II. Photomicrographs of sections through fornix and ventral psalterium of goldthioglucose treated CBA mice. CC, corpus callosum; LV, lateral ventricle; F, fornix; VP, ventral psalterium; AH, Ammon's horn; PH, primordial hippocampus; L, lesion.

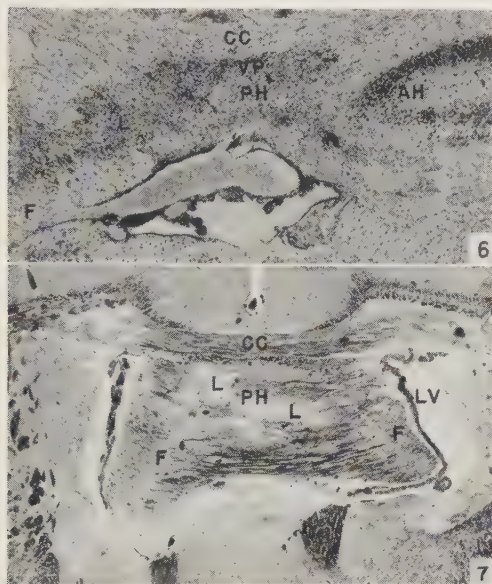


FIG. 6. Lesion of fornix and ventral psalterium associated with marked wt gain. Dose: .35 mg/g body wt. Duration of experiment: 15 days. Stain: Toluidin Blue. $\times 50$.

FIG. 7. Lesion of primordial hippocampus, ventral psalterium and fornix associated with marked wt gain. Dose: .35 mg/g body wt. Duration of experiment: 2 days. Stain: Weil's myelin. $\times 30$.

specimens. Gliosis was characteristic in animals allowed to survive 15 and 19 days after injection of goldthioglucose.

Discussion. It appears that the difference in response of CBA and C₅₇BL mice to goldthioglucose in terms of weight gain is not determined by mere presence or absence of lesions in the hypothalamus, but by extent of involvement of critical hypothalamic areas. Further investigation is needed to determine whether the "critical areas" are the postulated glucoreceptor center(7) or other regions. It is possible that the limbic lesions, which are being studied further in this laboratory relative to induced obesity are related to the

greater increase in weight gain shown by CBA mice.

Since lesions of variable and predictable size can be induced in the hypothalamus by altering the dose of injected goldthioglucose, opportunity is provided to study localization of hypothalamic function.

Summary. The response to goldthioglucose-induced obesity differs in two genetically independent inbred strains of mice (CBA and C₅₇BL). This difference in response to a specific dose is associated with degree of hypothalamic damage, more extensive in CBA than in C₅₇BL mice, the former becoming more obese than the latter. Even when hypothalamic lesions comparable to those induced in very obese CBA mice are obtained by relatively large doses of goldthioglucose in C₅₇BL mice, weight gain is approximately one-half of that appearing in CBA's. Lesions of the fornix also occurred in CBA but not in C₅₇BL mice; these lesions may be related to the greater weight increment in CBA mice. A further possibility is a genetically controlled (peripheral) metabolic difference in reaction to hypothalamic lesions in these 2 inbred strains.

The goldthioglucose used was obtained through the courtesy of Dr. Robert W. Burlew and the Schering Corp., Bloomfield, N. J.

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Received February 18, 1957. P.S.E.B.M., 1957, v95.

Effect of Glucagon on Alimentary Lipemia.* (23362)

MARGARET J. ALBRINK, JAMES R. FITZGERALD AND EVELYN B. MAN
(Introduced by Philip K. Bondy)

Department of Internal Medicine, Yale University School of Medicine, New Haven, Conn.

Recent investigations concerning interaction of carbohydrate and fat metabolism have suggested a relationship between the magnitude of alimentary lipemia and the status of glucose metabolism. The rise of particulate serum triglycerides (neutral fat) following ingestion of a fat meal by individuals who have fasted for 15 hours can be diminished or abolished by concomitant feeding of glucose(1). The hypothesis was set forth that any factors stimulating carbohydrate metabolism would reduce the degree of alimentary lipemia. Glucagon, like glucose, causes hyperglycemia, and increased peripheral uptake of glucose as great as(2) or greater than(3,4) that caused by doses of glucose which produce comparable arterial glucose concentrations. The effect of glucagon on alimentary lipemia was therefore made the subject of the present investigation.

Methods. The subjects were 6 male medical students and staff members between the ages of 21 and 30 who were free from known disease. The subjects were requested to eat or drink nothing but water after supper. On the morning of the experiment a preliminary blood sample was drawn, and the subjects were then fed a test meal containing 22 g protein, 60 g fat, and 42 g carbohydrate. The exact composition of this meal has been reported elsewhere(1). Two or 2½ hours after breakfast another blood sample was taken, and 0.03 mg glucagon†/kg body weight

(about 2 ml) diluted to 10 ml with physiological saline was injected intravenously. Glucagon was given just prior to the expected peak in concentration of serum triglycerides which usually occurs at about 3 hours with the fat meal used. In addition to glucagon, 2 subjects (RF, WA) received oral glucose in lemonade, as outlined in the next section. As a control, 3 subjects were given the same breakfast without glucagon on a separate occasion, 2 of them receiving 10 ml intravenous saline in place of the glucagon. However, an acute change in concentration of triglycerides in response to glucagon was thought to be of greater significance than a comparison of the 3-hour concentration on 2 different occasions with and without glucagon, because of variations in response of the same individual to the fat meal from time to time.

Sera obtained at intervals before and after breakfast and glucagon injection were analyzed for lipids including total fatty acids, total cholesterol, and lipid phosphorus, by methods which have been reported previously(5). Triglycerides were calculated from these data. In the sera of 2 subjects, the nonesterified fatty acids (NEFA) were analyzed by a modification(1) of the method of Dole(6). About an hour lapsed between drawing the blood and precipitating the serum in heptane and isopropyl alcohol for the NEFA determination. Blood sugar was determined by the Nelson-Somogyi method(7).

Results. Since no consistent change occurred in serum cholesterol and only an occasional slight rise occurred in the lipid phosphorus fraction after the fat meal with or without glucagon, the results reported will be confined to those fractions responding to glucagon: the triglycerides, blood sugar, and NEFA. A representative study is shown in Fig. 1. In 4 of the 6 subjects, glucagon given 2 to 2½ hours after the fat meal caused, within ½ hour, an 18 to 34 mg% increase in

* Aided in part by grants from the U. S. Public Health Service, and from the American Cancer Society.

† The glucagon kindly supplied by Dr. W. R. Kirtley, came from Lilly lot No. 208-158 B-214, containing 0.95 mg/ml of protein, and CT-800 glucagon lot No. P-60076, containing 1 mg protein/ml. Both lots were approximately 50% pure based on potency of crystalline glucagon (0.2 µg/kg of body weight injected intravenously into anesthetized cats causes a maximum blood sugar rise of 30 to 40 mg% 10 to 15 minutes following injection). Insulin content was less than 0.05 U/ml.

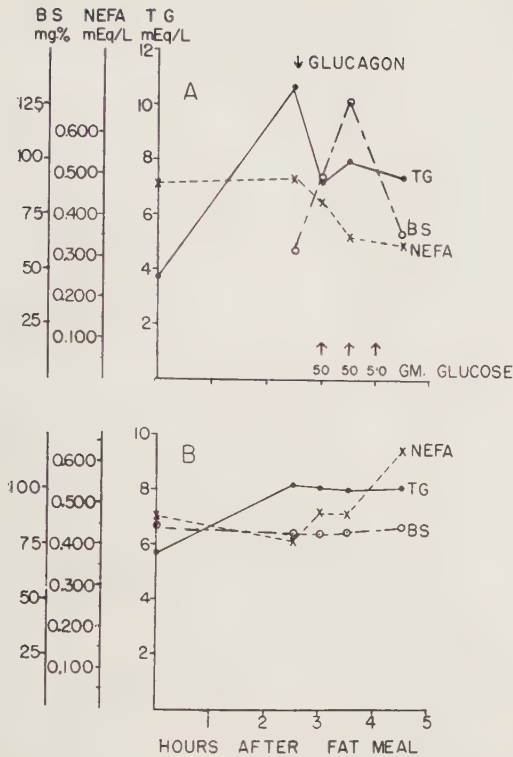


FIG. 1. Blood sugar (BS) serum nonesterified fatty acids (NEFA) and serum triglycerides (TG) of subject WA at intervals after a fat meal with (A) and without (B) glucagon.

blood sugar, and a reduction in concentration of serum triglycerides. The latter had risen from a mean fasting level of 4.1 mEq/L to 7.4 mEq/L at the time of glucagon injection, and decreased an average of 1.8 mEq/L $\frac{1}{2}$ hour following glucagon. There was visible clearing of the serum at this time, when the peak of alimentary lipemia would ordinarily have occurred. Intravenous injection of saline alone instead of glucagon was followed by a further rise in triglyceride concentration in 2 control studies.

Of the 2 exceptions, one (RM) had displayed no alimentary lipemia $2\frac{1}{2}$ hours after the fat meal at the time of glucagon injection, and although his blood sugar concentration increased $\frac{1}{2}$ hour after glucagon, there was no decrease in his serum triglyceride concentration (Fig. 2). The other exceptional subject failed to respond to glucagon with either a rise in blood sugar or a fall in triglyceride concentration. The latter subject was given

the test meal on another occasion, but instead of glucagon he received 150 g glucose in divided doses 1 hour, and $\frac{1}{2}$ hour before, and $\frac{1}{2}$ hour after breakfast, treatment known to abolish alimentary lipemia in most individuals(1), but despite this his serum triglyceride concentration rose 3 hours after the fat meal. On a third occasion he was fed the fat meal with the same amount of added glucose as on the second occasion, this time followed also by injection of glucagon 2 hours after the meal. This combination of glucose and glucagon effectively abolished the 3-hour peak in triglyceride concentration, although glucagon still caused no rise in blood sugar.

The NEFA measured in 2 of the subjects are shown graphically in Fig. 1 and 2 together with the triglyceride and blood sugar concentrations. Glucagon produced a rise in blood sugar and a fall in concentration of NEFA of both subjects. One normal subject (WA) was given oral glucose beginning one-half hour after the glucagon injection, in quantities indicated in Fig. 1. This prolonged the effect of glucagon on serum triglycerides and NEFA.

Discussion. The physiological influence of glucagon on carbohydrate metabolism is similar to that of glucose administration: by mobilization of liver glycogen it causes a temporary rise in blood sugar concentration. Although glucagon has been thought by some to decrease peripheral uptake of glucose (reviewed by Cardillo and Bondy(8)) recent experiments indicate that it does not interfere

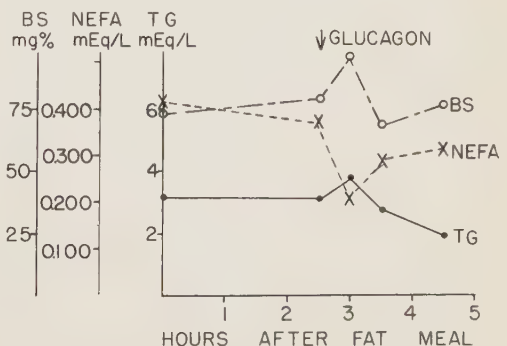


FIG. 2. Effect of glucagon on blood sugar, serum nonesterified fatty acids, and serum triglycerides of subject RM at intervals after a fat meal. Symbols same as in Fig. 1.

with peripheral uptake of glucose(2) and may even cause a greater peripheral uptake of glucose than does a hyperglycemia of similar degree evoked by intravenous glucose(3,4). Our study demonstrates that glucagon also has an effect on lipid metabolism: it diminishes alimentary lipemia and causes a fall in concentration of plasma NEFA. These effects are identical with the effects of glucose administration on alimentary lipemia(1) and on NEFA during fasting(6,9) or after a fat meal(1). The similarity of action of glucose and glucagon on lipid metabolism strengthens the view that glucagon increases rather than inhibits peripheral glucose utilization.

The effectiveness of glucagon would seem to depend on availability of glucose. One subject who experienced no rise in blood sugar also displayed no fall in serum triglycerides following glucagon. When he was prefed glucose his alimentary lipemia was then abolished by glucagon although both glucagon and glucose alone were ineffective. Availability of liver glycogen is a limiting factor in ability of glucagon to induce hyperglycemia(3).

Experimental(10,11) and clinical(12) observations suggest that the pancreas has a role in removal of triglyceride particles from the blood stream. The possible significance of the present study to those observations is not clear. At present it can be concluded only that glucagon, like glucose, causes an acute reduction of particulate triglycerides and of NEFA of plasma. Although other mechanisms must be considered, this action of glucagon may be entirely secondary to the large amount of glucose mobilized or diverted from the liver and thus made available to stimulate peripheral carbohydrate metabolism.

Summary. 1. In 4 of 6 normal subjects, the intravenous injections of 0.03 mg of glu-

cagon per kg body weight $2\frac{1}{2}$ hours after the ingestion of a standard fat breakfast caused a reduction of serum triglyceride concentration either toward or to the postabsorptive levels within half an hour. Of the 2 exceptions one failed to display any rise in concentration of triglycerides following the fat meal, prior to glucagon injection. The second failed to have a hyperglycemic response to glucagon. The rise in concentration of his serum triglycerides could be prevented by glucagon only when he was fed additional glucose with the fat meal. 2. Concentration of nonesterified fatty acids, measured in the sera of 2 subjects decreased following glucagon administration. 3. It was concluded that the effect of glucagon on serum lipids is secondary to its hyperglycemic effect.

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Received May 15, 1957. P.S.E.B.M., 1957, v95.

Preparation of Partially Purified Porcine Intrinsic Factor. (23363)

KENNETH C. ROBBINS AND JANE SHIELDS

Research Division, Armour and Co., Chicago, Ill.

The preparation of highly purified porcine intrinsic factor has been described(1,2,3). The active principle appears to be mucoprotein in nature. The methods described for the preparation of porcine intrinsic factor use whole porcine stomach as the starting material and are detailed and complicated. This report describes a new and simple method for preparing highly purified porcine intrinsic factor from porcine stomach pyloric mucosa. The evaluation of this material both clinically and in the urinary excretion test will be discussed. Experiments on the further purification of porcine intrinsic factor on cellulose-ion exchangers will be described.

Methods. *A. Preparation of intrinsic factor.* To each kg of fresh, frozen, ground porcine stomach pyloric mucosa was added 2 l of ice-cold water. The pH of the suspension was adjusted to 3.5 with 8N HCl and the mixture was stirred overnight at 2°C. The bulk of the residue was removed by filtration through cheesecloth. The remaining suspension was further clarified by centrifugation at 4000 rpm, at 2°C, for 1 hour. The extract of intrinsic factor was concentrated by either vacuum distillation or lyophilization. The solids content at this point, as determined by lyophilization, was 30 g/kg tissue. The concentrate (pH 4.0) was adjusted to 7% total solids and to pH 5.2 with 2N NaOH. A one molar sodium acetate-acetic acid buffer, pH 5.2, was added to this concentrate to give an ionic strength of 0.15 (sodium acetate) at a solids concentration of 6% at 2°C. Ninety-five % ethanol at -20°C was added to this extract to final concentration of 50% at -5°C. After standing overnight at -5°C, the precipitate was removed by centrifugation at 4000 rpm, at -5°C, for 30 minutes. It was suspended in ice-cold distilled water (4 x precipitate volume) and stirred overnight at 2°C. The solution of intrinsic factor (PI) was lyophilized. The yield, after lyophilization, was 5-6 g/kg tissue. Dialysis of intrinsic factor

PI before lyophilization resulted in a loss of 60% of the solids.

B. Clinical evaluation. A number of intrinsic factor PI preparations were evaluated for clinical activity using a hematologic method in carefully selected pernicious anemia patients in relapse. The tests were carried out by physicians in accordance with recommendations of the Anti-Pernicious Anemia Board of the U.S. Pharmacopoeia(4). The patients were not used for more than one test. Each patient was hospitalized throughout the entire 3-week period of assay, with red blood cell counts and reticulocyte counts taken either daily or 3 times weekly. Thirty-five mg of intrinsic factor preparation PI was mixed with 15 µg crystalline vit. B₁₂ and the mixture was given orally, suspended in water or orange juice, an hour or more before breakfast. The average red cell count for 2 or 3 days before therapy and the red blood cell count at the 21-day level were compared with the expected red blood cell response in the U.S. Pharmacopoeia recommended tables(5). The percent response was calculated by dividing the 21-day red blood cell response by the expected red blood cell response. The percentage of reticulocyte response was calculated in a similar way using U.S. Pharmacopoeia recommended tables(6). The clinical evaluation of intrinsic factor PI is summarized in Table I. The clinical data on 4 preparations indicated that a *full* red blood cell response (90-100% of U.S.P. response expected in 21 days) occurred in the majority of cases. The excellent clinical response in practically all cases indicated that the daily dose used, namely, 35 mg intrinsic factor PI and 15 µg vit. B₁₂, could be defined as one U.S.P. oral unit.* The dialyzed and lyophilized intrinsic factor PI (IV-D) appears to be fully active in a daily dose of about 15 mg intrinsic factor and 15 µg B₁₂.

* Intrinsic factor—vitamin B₁₂ preparation used in Biopar Forté, Armour Laboratories.

TABLE I. Clinical Evaluation of Intrinsic Factor PI.*

Preparation No.	Intrinsic factor dose, mg	B ₁₂ dose, μ g	Reticulocytes				Red blood cells, millions/mm ³			Clinical response,† % of expected R.B.C.
			Initial, %	Peak, % at Day	% of expected ret. response		Initial	At 14 days	At 21 days	
I	35	15	.3	16.4	5	46	1.17	3.14	4.08	100
"	"	"	.5	18.1	8	60	1.40	2.49	3.08	93
"	"	"	5.6	20.7	8		1.73		2.41	42
"	"	"	1.9	8.2	8	71	2.45	2.96	3.88	100
"	"	"	.6	11.1	8	31	1.37	2.21	3.25	100
"	"	"	1.0	24.2	7	100	1.70		3.60	100
II	"	"	4.0	15.6	7	53	1.49	2.00	2.62	64
"	"	"	2.9	16.6	8	55	1.43	3.00	3.46	100
"	"	"	.9	29.8	6	87	1.25		2.87	82
"	"	"	.9	30.6	9	84	1.20	2.80	3.70	100
III	"	"	.7	7.5	8	30	1.49	2.46	3.25	100
"	"	"	.6	22.9	8	100	2.09	3.46	3.80	100
"	"	"	.8	19.4	6	62	1.35	2.10	2.80	80
"	"	"	.7	10.0	12	50	1.90	3.20	2.94	65
"	"	"	2.2	26.6	9	80	1.30	2.80		100 (14 days)
"	"	"	2.0	14.5	10	76	1.96	2.74	2.62	51
"	"	"	2.8	14.8	8	97	2.20		3.29	71
IV	"	"	.8	16.2	8	89	2.00		3.56	100
IV-D	15	"	.4	23.7	8	78	1.34	1.71	2.46	78
"	"	"	3.4	23.0	8	78	1.32	2.60	3.05	97

* One U.S.P. unit of intrinsic factor and vit. B₁₂ is equivalent to amt of intrinsic factor which, when given with 15 μ g B₁₂ in a single daily oral dose to a pernicious anemia patient in relapse, will give a full red blood cell response (90-100% of expected response) in 14 or 21 days according to U.S.P. calculations(4,5).

† Based on red blood cell response in 21 days.

C. *Urinary excretion test.* The technic used in the 24-hour urinary excretion test has been described(7). The test used to evaluate intrinsic factor PI involved the use of an oral dose of 1 μ g vit. B₁₂ Co⁶⁰ and an intramuscular dose of 1000 μ g non-radioactive vit. B₁₂. The results of the urinary excretion tests on intrinsic factor PI-IV are summarized in Table II. The 27 control tests gave a urinary excretion of $1.7 \pm 1\%$. The 14 subjects receiving 15 mg intrinsic factor PI (0.43 U.S.P. unit) showed a urinary excretion of $11.0 \pm 4.6\%$ and the 10 subjects receiving 50 mg intrinsic factor PI (1.43 U.S.P. units) showed a urinary excretion of $18.1 \pm 5\%$. The patients receiving 50 mg intrinsic factor PI excreted approximately 0.18 μ g vit. B₁₂, which appears to be close to the maximum excretion of vit. B₁₂ when using a 1 μ g vit. B₁₂ oral dose. The amount absorbed is probably 3-4 times the amount excreted(7,8). Therefore, excretion of 0.18 μ g vit. B₁₂ corresponds to an absorption of about 0.54-0.72 μ g vit. B₁₂. Since the maximum absorption which could be expected from 1 μ g vit. B₁₂ oral dose in normal subjects

is about 70-90%(9), the 50 mg intrinsic factor PI dose gives close to maximum absorption of vit. B₁₂. Three subjects previously tested with 50 mg intrinsic factor PI were then tested with 350 mg intrinsic factor PI (10 U.S.P. units). They showed a urinary excretion of $14.7 \pm 1.5\%$. Extraordinarily high or unphysiological doses of intrinsic factor appear to depress absorption of vit. B₁₂ Co⁶⁰. Our data show that less than 0.5 U.S.P. unit of intrinsic factor PI will give significant and measurable urinary excretion of vit. B₁₂ and that 1.5 U.S.P. units of intrinsic factor PI will give close to maximum excretion of vit. B₁₂. These results are in contradiction to those reported by Williams *et al.*(10) who found that 2-3 U.S.P. units of intrinsic factor are needed to give significant urinary excretion values. The difference may be due to the dosage of vit. B₁₂ Co⁶⁰ used (1 μ g *vs.* 2 μ g) or to the intrinsic factor preparations used in the 2 studies. It is more likely that the discrepancy is due to the incorrect assignment of potency by Williams *et al.*(10) to their standard preparation. It is obvious from their pub-

TABLE II. Urinary Excretion Tests Using Intrinsic Factor PI-IV and 1 μ g Vit B₁₂ Co⁶⁰ in 27 Patients.

% B ₁₂ excreted			
Intrinsic factor (mg)			
0	15	50	350
2.1	6.9		
1.1	6.3		
3.8	14.1		
1.4	6.7		
.2			
1.0	13.0		
1.8			
2.4	12.6		
2.5	22.7		
.7	12.5		
1.7	9.4		
.8			
1.9	9.3		
2.2	7.5		
2.0		22.0	
3.7		21.0	14.2
.7		8.5	13.5
1.5		22.1	16.4
2.0		20.6	
.2		13.2	
1.2		13.9	
2.6		20.9	
2.4		23.6	
2.8		15.5	
.3	7.7		
1.0	16.4		
.7	9.6		
* 1.7 $\pm$ 1.0	11.0 $\pm$ 4.6	18.1 $\pm$ 5.0	14.7 $\pm$ 1.5

* Mean $\pm$ S.D.

lished data(10) that the intrinsic factor preparation used as the standard did not show full potency for unitage assigned. The 2-3 U.S.P. units used in the urinary excretion tests are probably less than one unit if the U.S. Pharmacopoeia recommended tables(5) are used.

D. Further purification using cellulose-ion exchangers. Intrinsic factor PI-IV was fractionated on a DEAE-cellulose ion exchanger (11) at pH 7.0 using gradient elution. The results of the experiment are summarized in the elution diagram in Fig. 1. The fractions were assayed in the urinary excretion test. The activity was adsorbed and eluted in fractions Nos. A₂, A₃ and A₄ (Table III). The unadsorbed fraction, A₁, was inactive. Since 60% of the solids are lost on dialysis, the recovered fractions account for approximately all of the non-dialyzable solids. The estimated activity of the adsorbed fractions was 12, 9 and 10 mg/U.S.P. unit, respectively.

The fractions were 3-4 times as potent as the starting preparation and about 1.5 times as potent as the preparation obtained on dialysis of intrinsic factor PI (IV-D) which had an activity of about 15 mg/U.S.P. unit (Table I). There was a loss in recoverable activity. A comparison of intrinsic factor PI-IV and PI-IV-D in the urinary excretion test was then made and the results are summarized in Table III. The estimated U.S.P. potency of intrinsic factor PI-IV-D from the urinary excretion test is about 15 mg, which is about the same as that found in the standard U.S.P. assay (Table I). Fraction No. A₄ (Fig. 1), containing the bulk of the activity, was re-chromatographed on a CM-cellulose exchanger(11) at pH 5.4 using gradient elution. The gradient was produced with 0.01 M acetate buffer, pH 5.4, and 0.5 M NaCl. Two

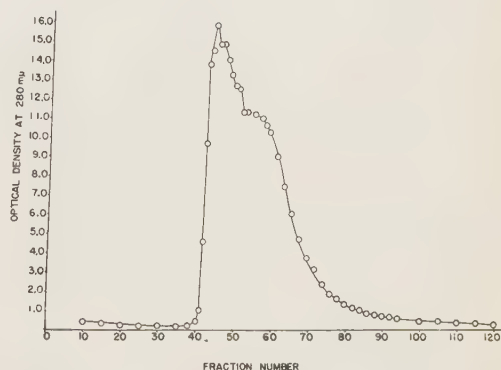


FIG. 1. Effluent diagram: 10 g intrinsic factor PI were dissolved in 300 ml 0.005 M phosphate buffer, pH 7.0. pH of the solution was adjusted to 7.0. Solution was dialyzed against 8 l of phosphate buffer for 48 hr at 1°C, was clarified by centrifugation since a precipitate appeared during dialysis. Supernatant solution was passed through a DEAE-cellulose column (41 $\times$ 2.5 cm) (Solka-Floc BW; .50 mEq/g) which had been equilibrated with 0.005 M phosphate buffer, pH 7.0. Column was washed with buffer and the wash collected in fractions 1-10. Gradient was begun at fraction 10 and produced by continuous introduction of .1 M Na₂HPO₄ + .5 M NaCl into a constant volume reservoir containing 100 ml of .005 M phosphate buffer, pH 7.0. Fraction volume was 6 ml and flow rate was 1.5 ml/min. Experiment was carried out at 25°C. Ordinate is optical density at 280 mμ and represents protein. Fraction pools 1-10 (Fraction No. 1), 42-46 (Fraction No. 2), 47-53 (Fraction No. 3), and 54-84 (Fraction No. 4) were dialyzed against distilled water at 1°C for 48 hr and lyophilized. Weights of fractions were as follows: No. A₁—1.50 g; No. A₂—.36 g; No. A₃—.56 g; No. A₄—1.20 g.

TABLE III. Urinary Excretion Tests on Various Fractions and Subfractions of Intrinsic Factor PI-IV.

for PI-IV.										
Patient No.	No intrinsic factor (control), % of B ₁₂ excreted	Intrinsic factor PI-IV (standard) mg % of B ₁₂ excreted	Further fractionation of intrinsic factor PI-IV							
			Fraction	Yield*		mg	% of B ₁₂ excreted	Estimated U.S.P. units†	Purification factor‡	
1	1.4	15	6.7	A ₁	15	% of PI-IV	5	.4	0	0
2	2.0	50	22.0	A ₂	3.6	"	"	9.5	.4	3
"	"	"	"	A ₃	5.6	"	"	12.7	.6	4
"	"	"	"	A ₄	12	"	"	11.3	.5	3.5
3§	1.3	15	3.3	PI-IV-D	40	"	6	4.1	.5	3
"	"	"	"	"	"	"	15	7.1	.9	2
4	2.0	50	20.6	"	"	"	6	12.0	.6	3.5
5	1.1	15	6.3	B ₁	25%	of A ₄	5	6.4	.4	3
6	1.6	"	11.1	B ₂	52	"	"	9.4	.4	2.5
				C ₁	21%	of PI-IV				
7	1.7	"	9.4	C ₂	19	"	"	12.6	.6	4
"	"	"	"	D ₁	27%	of C ₂	"	6.2	.3	2
8	1.9	"	9.3	D ₂	11	"	"	11.5	.5	3.5
9	2.5	"	22.7	D ₃	10	"	"	5.2	.1	.5
10	.7	"	12.5	D ₄	24	"	"	.7	0	0

* % of starting material wt.

† Est. U.S.P. = $K \times \frac{\text{sample \%}}{\text{standard \%}}$ where $K = 1.0$ for 35 or 50 mg; $K = .43$ for 15 mg of standard.

‡ Purification factor = Est. U.S.P. $\times \frac{35}{\text{mg of sample}}$.

§ Patient tested with 2 rather than 1 μg B₁₂Co⁶⁰.

|| No standard test done; value taken from series in Table II.

chromatographic peaks were obtained, one unadsorbed (B₁) and the other adsorbed and eluted (B₂). The activity appeared in both fractions (Table III), with no purification over the starting material.

In a third experiment, intrinsic factor PI-IV was fractionated on the DEAE-cellulose exchanger at pH 5.4 using gradient elution. The gradient was produced with 0.01 M acetate buffer, pH 5.4, and 0.5 M NaCl. Two chromatographic peaks were obtained, one unadsorbed (C₁) and the other adsorbed and eluted (C₂). The activity was adsorbed at pH 5.4 (Table III) and eluted in a single fraction, C₂. The estimated potency of fraction No. C₂ is about 9 mg/U.S.P. unit. The unadsorbed fraction was not assayed since in a preliminary experiment the unadsorbed fraction was inactive. Fraction No. C₂ was rechromatographed on DEAE-cellulose at pH 4.7 using gradient elution. The results of this experiment are summarized in the elution diagram in Fig. 2. The bulk of activity was unadsorbed (Table III). The most active fraction had about the same potency as the

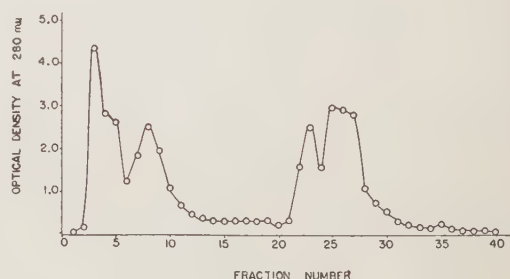


FIG. 2. Effluent diagram: 500 mg intrinsic factor Fraction No. 2 was dissolved in 10 ml .01 M acetate buffer, pH 4.7. Solution was dialyzed against 200 ml of the acetate buffer for 48 hr at 1°C. It was passed through a DEAE-cellulose column (37 $\times$ 1.1 cm) which had been equilibrated with 0.01 M acetate buffer, pH 4.7. Column was washed with buffer and gradient was begun at fraction 15 and produced by adding 0.01 M acetate buffer, pH 4.7, plus 0.5 M NaCl into a constant volume reservoir containing 100 ml 0.01 M acetate buffer, pH 4.7. Fraction volume was 6 ml and flow rate was 0.75 ml/min. Experiment was carried out at 25°C. Ordinate is optical density at 280 mμ and represents protein. Fraction pools 3-6 (Fraction No. 1), 7-11 (Fraction No. 2), 22-24 (Fraction No. 3), and 25-29 (Fraction No. 4) were dialyzed against distilled water at 1°C for 48 hr and lyophilized. Weights of fractions were as follows: No. D₁—138 mg; No. D₂—54 mg; No. D₃—50 mg; No. D₄—120 mg.

TABLE IV. Carbohydrate Analysis of Intrinsic Factor Fractions.

Fraction No.	Intrinsic factor activity, mg/U.S.P. unit	Carbohydrate as	
		Mannose, %	Galactose, %
PI-IV	35	5.4	5.8
PI-IV-D	15	8.2	10.6
A ₁	Inactive	12.8	16.5
A ₂	12	8.6	11.1
A ₃	9	6.5	8.4
A ₄	10	4.2	5.4
B ₁	12	6.1	7.9
B ₂	14	2.2	2.7
C ₁		12.8	16.4
C ₂	9	6.3	8.1
D ₁	18	10.9	14.0
D ₂	10	3.1	3.9
D ₃	70	7.7	10.0
D ₄	Inactive	6.7	8.6

starting material. The number of peaks obtained in this experiment indicated that fraction No. C₂ was heterogeneous. There was also a considerable loss in recoverable activity. The active molecule may have split into more than one component on further purification or some prosthetic group may have been split off. The most active fraction obtained by the DEAE-cellulose chromatographic method had an estimated potency of 9 mg per U.S.P. unit.

The various fractions obtained were analyzed for carbohydrate by the method of Gurin and Hood(12). The results are summarized in Table IV. The 520/420 ratios indicate either mannose or galactose. There appears to be some correlation between intrinsic factor activity and carbohydrate content since the most active fractions contained the smallest amount of carbohydrate.

Summary and conclusions. A simple method is described for preparation of highly purified intrinsic factor PI from porcine stomach pyloric mucosa. In pernicious anemia patients in relapse, 35 mg of intrinsic factor PI when given with 15 μ g vit. B₁₂ is defined as one

U.S.P. oral unit. Dialysis of intrinsic factor PI gives a material with a potency of about 15 mg/U.S.P. unit. In the urinary excretion test, one-half U.S.P. unit of intrinsic factor PI gives significant and measurable urinary excretion of vit. B₁₂ Co⁶⁰ when given orally with 1 μ g vit. B₁₂ Co⁶⁰. Approximately 1.5 U.S.P. units of intrinsic factor PI will give maximum urinary excretion of vit. B₁₂ Co⁶⁰. Intrinsic factor PI can be further purified on a DEAE-cellulose ion exchanger to give a material with an estimated potency of 9 mg/U.S.P. unit.

The authors wish to thank W. F. White, Armour and Co., and Dr. W. R. Best, University of Illinois College of Medicine, for their assistance in carrying out the urinary excretion tests.

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Received May 23, 1957. P.S.E.B.M., 1957, v95.

Variations in Muscle Electrolyte Composition Due to Sampling and to Aging.* (23364)

MALCOLM A. HOLLIDAY,[†] WILLIAM E. SEGAR, ARLENE LUKENBILL, RUBY MARIE VALENCIA, AND ANNA MAY DURELL (Introduced by Robert Klein)

Department of Pediatrics, Indiana University Medical Center, Indianapolis.

The determination of potassium in muscle has been widely used in studies of potassium deficiency and factors relating to it. The potassium of muscle has been expressed in a variety of ways using different components of muscle as a basis of reference. These have included dry fat free muscle solids(1,2,3) and calculated intracellular water(4,5). The expression in terms of dry muscle is an effort to relate the potassium to muscle protein. This has been modified to be expressed in terms of collagen-free nitrogen(6). The expression in relation to muscle solids has the advantage of being a simply obtained analytic figure independent of calculations. It has, by now, gained widespread acceptance. On the other hand, expressing muscle potassium in terms of its concentration in intracellular water may represent a value more meaningful in physico-chemical relations and does not reflect changes in muscle potassium resulting from variations in intracellular fluid volume in relation to muscle solids. This method of expression, however, is dependent on the assumption that the chloride space may be used as an index of extracellular volume.

The following report compares potassium content expressed in these 2 ways of muscle taken from the thigh to that obtained from the back in 2 groups of rats. The first group of rats consisted of normal 10-12 week old animals. The second group consisted of similar rats, allowed to grow an additional 7 weeks on a standard "chow" diet. Thus sampling from 2 different sites was compared using each method of expression in young mature rats and again in older larger rats. In addition, differences due to growth as reflected by changes noted in either sample could be compared. In this way validity of sampling and method of expression could each be explored.

Methods. Sixteen rats were fed lab chow from weaning. At approximately 10-12 weeks of age the first group of 8 were sacrificed. The second group of 8 were continued on the diet for 7 additional weeks. Group A averaged 255 grams and Group B averaged 250 g initially. Group B gained to a weight of 375 g in the ensuing 7 weeks, a gain of 125 g. The animals were killed by removing 8-12 cc blood from the abdominal aorta. Muscle from each rat was obtained separately from the thigh and from the anterior paravertebral region and was placed in separate weighing bottles. In the case of the thigh muscle, duplicate analyses on aliquots of differing weight were obtained. There was insufficient muscle for duplicate determinations on the paravertebral or back muscle. The method of analysis has been previously described(7) and was modified in only one respect. Elution of electrolyte from the muscle using nitric acid was carried out on a sample not previously fat extracted rather than on a fat extracted aliquot since slightly lower values were sometimes obtained in the latter instance. Fat extraction was done on separate aliquots of dry muscle. The method of analysis results in analytical values being directly determined in relation to muscle solids. Where duplicate analyses were done the per cent standard deviation between duplicates was as follows: Na $\pm$ 1.2%; Cl $\pm$ 1.3%; K $\pm$.7%.

Results. Data comparing mean values for back and leg for each group are given in Table I. Differences between back and leg were observed in the case of chloride and potassium expressed in relation to fat free dry solids (FFDS) of muscle. In the young group these differences were statistically significant for chloride as well as for potassium, while in the older group the statistical significance could be demonstrated only in the case of potassium. The directions of change were the same in both groups of animals. Chloride was

* Aided by a grant from Riley Memorial Assn.

[†] Present address, Children's Hospital of Pittsburgh, Pa.

TABLE I. Data of Young and Old Groups Comparing Thigh and Back Samples.

	Expressed in terms of 100 g dry fat free solids										Per kg cell water		
	(H ₂ O) _m , g	(Cl) _m , mEq	(Na) _m , mEq	(K) _m , mEq	(Na) _i , mEq	(H ₂ O) _e , g	(H ₂ O) _i , g	[Na] _i	[K] _i	[Na + K] _i	[Na] _i	[K] _i	[Na + K] _i
Young—Thigh Back	340 ± 6	5.96 ± .41	9.51 ± .60	45.78 ± 1.16	1.54 ± .22	53.5 ± 4.5	286 ± 5	5.3 ± .7	160 ± 6	165 ± 6	5.3 ± .7	160 ± 6	165 ± 6
	345 ± 15	5.22 ± .40	9.80 ± 1.37	48.83 ± .68	2.81 ± .77	47.0 ± 4.2	298 ± 10	9.4 ± 2.4	164 ± 6	173 ± 5	9.4 ± 2.4	164 ± 6	173 ± 5
Old—Thigh Back	322 ± 4	4.85 ± .26	10.62 ± .60	42.58 ± .83	4.20 ± .52	42.0 ± 2.8	280 ± 7	15.0 ± 1.9	152 ± 3	167 ± 4	15.0 ± 1.9	152 ± 3	167 ± 4
	327 ± 10	4.54 ± .40	9.89 ± .78	44.93 ± 1.68	3.86 ± .76	39.6 ± 4.4	287 ± 5	13.5 ± 2.7	157 ± 5	170 ± 6	13.5 ± 2.7	157 ± 5	170 ± 6
Mean differences between samples from thigh and back													
Young—Δ	-5	+74	-29	-3.05	-1.27	+6.5	-12	-4.1	-4	-8	-4.1	-4	-8
p*	ns	<.01	ns	<.001	<.05	<.01	ns	<.05	ns	<.05	<.05	ns	<.05
Old—Δ	-5	+31	+73	-2.35	+34	+2.4	-7	+1.5	-5	-3	+1.5	-5	-3
p*	ns	ns	ns	<.01	ns	ns	ns	ns	ns	ns	ns	ns	ns
Mean differences between young and old using thigh and back respectively													
ΔT	-18	-1.11	+1.11	-3.20	+2.66	-11.5	-6.8	+9.7	-8	+2	+9.7	-8	+2
p*	<.01	<.01	<.01	<.001	<.01	<.01	ns	<.01	<.01	ns	<.01	<.01	ns
ΔB	-18	-68	+09	+3.90	+1.05	-7.4	-11	+4.1	-7	-3	+4.1	-7	-3
p*	<.01	<.01	ns	<.01	<.05	<.01	<.05	<.05	<.05	ns	<.05	<.05	ns

* Probability using Student's "T" test for significance.

ns = not significant.

higher in thigh muscle than in back muscle, resulting in a larger calculated extracellular space.

Potassium, however, was lower in thigh muscle than it was in back for both young and old groups (-6.7%; -5.5% respectively). This difference was not accompanied by a parallel difference in total water (-2.4%; +1.6%), but was accompanied by a parallel difference in calculated intracellular water (-4.2%; -2.5% respectively) to potassium was observed. The difference in intracellular water did not have the same statistical significance as the difference in potassium per 100 g FFDS. However, when potassium was expressed in terms of its concentration in intracellular water, the difference in potassium noted above is no longer apparent.

In the young group only, a difference between thigh and back in the calculated values of intracellular sodium by either method of expression was also observed. This was not true in the older group, and its significance in the young group is not apparent.

The data comparing muscle composition of the young to the older group using data from either sample is given in Table I. A decrease in potassium associated with growth and aging could be demonstrated using data from thigh or from back for comparison. The decline in potassium with growth was evident by either method of expression, *i.e.*, in relation to muscle solids or expressed as concentration in intracellular water. These differences were accompanied by reciprocal increases in intracellular sodium such that the sum of sodium and potassium was not different in comparing young rats to old ones.

Discussion. The higher values of chloride and calculated extracellular volume in thigh muscle are very likely due to more connective tissue being present in thigh muscle. The samples from the thigh by simple inspection had more connective tissue. No collagen determinations were made to substantiate this.

The differences observed in the case of potassium in relation to muscle solids can be ascribed either to variations of potassium in relation to intracellular solids, to differences in the density of cell solids, or to differences in

solids from the extracellular phase to total muscle solids. The first 2 explanations imply some fundamental difference in muscle composition at the two sites. The third explanation is satisfied by postulating a difference in connective tissue in relation to muscle. If the observed differences noted in extracellular fluid between thigh and back are due to variations in connective tissue, then total muscle solids are increased in thigh muscle because of the increase in connective tissue. The lower value of potassium in relation to total muscle solids in thigh muscle might then represent an artifact due to an increase of connective tissue solids. The magnitude of connective tissue solids predicted by the difference in extracellular fluid is sufficient to account for these differences(8). The finding of a constant concentration of potassium in intracellular water lends support to this explanation.

This constancy of intracellular potassium concentration also strengthens the validity of those assumptions necessary to calculation of potassium concentration in intracellular water.

It is apparent from these data that studies relating muscle potassium values to cell solids should utilize samples from comparable sites. It is quite possible that long periods of potassium deficiency may result in greater variations in connective tissue or other muscle solids in relation to intracellular fluid as a consequence of anatomical changes in muscle from similar sites wrought by potassium deficiency. This would result in falsely low muscle potassium values when they are expressed in terms of muscle solids. Expressing potassium in terms of its concentration in intracellular water may be a more precise definition of a real decrease in muscle potassium despite the greater number of assumptions inherent in its calculation. If these changes are due solely to changes in connective tissue solids, correcting muscle solids for collagen may serve to obviate the difficulties cited here in using muscle solids as a basis of reference.

The changes in muscle potassium associated with growth and aging are in the same direction but are even more pronounced than those reported by Lowrey and Hastings(4). It is

pertinent to observe that increase in muscle sodium was equivalent to decrease in muscle potassium so that the sum of the 2 remains constant. This is in contrast to the initial decreases in potassium concentration observed early in the development of potassium deficiency(9). In the latter instance the initial decrease in muscle potassium is not accompanied by a significant increase in muscle sodium so that the sum of the 2 is substantially decreased.

Summary. 1) A difference in muscle potassium was demonstrated in samples from 2 different sites of the same animal when potassium was expressed in terms of fat free dry muscle solids. This difference did not exist when potassium was expressed in terms of its concentration in calculated intracellular water. We suggest that the discrepancy was related to differences in connective tissue or extracellular solids between the 2 samples, and that potassium expressed in terms of its concentration in intracellular water was the more meaningful expression. 2) A small but significant decrease in potassium associated with growth and aging was also noted. This difference was demonstrated expressing potassium in terms of muscle solids or intracellular water concentration. A reciprocal increase in calculated intracellular sodium was noted such that the sum of sodium and potassium remained constant.

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Received May 27, 1957. P.S.E.B.M., 1957, v95.

Pharmacological Studies with Pseudoreserpine and Raunescine, Two Alkaloids Isolated from *Rauwolfia canescens* Linn. (23365)

SOL ROTHMAN AND IDA MARIA TOEKES (Introduced by Georg E. Cronheim)
(With technical assistance of J. Cawthorne and R. Bitterman)

Research Division, Riker Laboratories, Los Angeles

A number of alkaloids have been isolated from *Rauwolfia canescens* Linn. Among these are deserpidine(1,2,3) raunescine and isoraunescine(4). Klohs *et al.* have isolated an alkaloid with chemical characteristics similar to raunescine and a new alkaloid, pseudoreserpine(5). Raunescine and pseudoreserpine were subjected to pharmacologic investigation for cardiovascular and sedative effects.

Methods. The sedative effect of the alkaloids in mice was determined by performance in roller cages(6). Raunescine and pseudoreserpine were administered intraperitoneally in varying doses to 189 and to 110 mice respectively, and the animals were tested 2 hours later. Dogs for cardiovascular testing were chosen at random and divided into groups of 5 animals regardless of sex or weight. Pseudoreserpine was given to one group at 0.5 mg/kg/day orally for 5 days. Raunescine was given orally to another group at 1 mg/kg/day for a like duration. The test methods have been described previously(7). The responses to epinephrine hydrochloride solution (1 and 3 μ g/kg i.v.) were recorded in both control and test runs. Following the test run, the vagi were sectioned bilaterally and the responses to bilateral carotid occlusion and right central vagus faradization were recorded. Pseudoreserpine or raunescine was given intravenously at 0.3 or 1 mg/kg to 4 urethanized and vagotomized dogs. Responses to the following standard series of tests were

observed: 3 and 6 μ g/kg epinephrine, 1 μ g/kg acetylcholine, 1 μ g/kg isopropyl-norepinephrine, 1 μ g/kg histamine, 1 minute tilting to 60°, bilateral carotid occlusion (30 sec.), and right central (2 volts, 15 sec.) and peripheral vagus stimulation (2 volts, to effect). The tests were repeated hourly for 5 hours. All drugs were administered intravenously; pressure from the femoral artery was recorded on an electrokymograph. Lack of material prevented additional testing in dogs.

Results. Both alkaloids produced visible "sedation" in mice and caused "fall-outs" from roller cages. The ED₅₀ (95% confidence limits) for pseudoreserpine was 11 (8-14) mg/kg and that for raunescine 7 (5-10) mg/kg. The slope functions were 2.7 (1.8-4.1) and 5.0 (2.5-9.7) respectively. There were no gross signs of ataxia. The corresponding figure for reserpine(8) was an ED₅₀ of 0.84 (0.62-1.10) mg/kg with a slope function of 2.1 (1.7-2.6).

After 5 days of oral medication the dogs exhibited sedation, diarrhea and prominence of the nictitating membrane, signs characteristic of treatment with a reserpine type alkaloid. The alkaloids reduced mean arterial pressure (MAP) and heart rate (HR) (Table I). After raunescine, MAP fell from 121 to 82 mm Hg, while HR decreased from 127 to 84 beats/min. Following pseudoreserpine MAP decreased from 117 to 89 mm Hg, with HR changing from 127 to 67 beats/min. No

TABLE I. Cardiovascular Effects in Dogs after 5 Days of Oral Medication. 5 dogs/series.

Alkaloid and dose in mg/kg	Heart rate* (beats/min.)		Mean art. pressure* (mm Hg)		Response to i.v. (1 μ g/kg)		Epinephrine† (3 μ g/kg)	
	Control	Test	Control	Test	Control	Test	Control	Test
Raunescine, 1.0	127 $\pm$ 22	84 $\pm$ 18	121 $\pm$ 22	82 $\pm$ 20	123 $\pm$ 26 +69 $\pm$ 16	88 $\pm$ 29 +114 $\pm$ 42	132 $\pm$ 28 +112 $\pm$ 17	86 $\pm$ 29 +144 $\pm$ 36
Pseudoreserpine, .5	127 $\pm$ 22	67 $\pm$ 15	117 $\pm$ 12	89 $\pm$ 10	112 $\pm$ 24 +55 $\pm$ 36	94 $\pm$ 38 +77 $\pm$ 24	101 $\pm$ 21 +83 $\pm$ 44	89 $\pm$ 29 +128 $\pm$ 20

* Heart rate and mean arterial pressure measured under pentobarbital anesthesia and expressed as mean ($\pm$ stand. dev.).

† Initial MAP ($\pm$ stand. dev.) + rise in pressure ($\pm$ stand. dev.).

TABLE II. Cardiovascular Responses in Dogs after Intravenous Administration in Acute Experiments (All Figures Refer to mm Hg).

Drug and dose in mg/kg	Test	Control	½ hr	1½ hr	2½ hr	3½ hr	4½ hr	5½ hr	8 hr
Raunescine, .3	MAP in mm Hg	106	106	110	121	126	120	122	
	Epi*	+4§	+24	+36	+55	—	—	—	
	CO†	+22	+11	+20	+49	+36	+42	+48	
	RCV‡	+122	+79	+58	+38	+28	+13	+10	
" , 1	MAP in mm Hg	96	70	62	74	88	80	77	
	Epi	+4	+20	+28	+46	—	—	—	
	CO	+8	-4	+5	+6	+10	+10	+15	
	RCV	+7	+6	+16	+8	+10	+9	+7	
Pseudoreserpine, .3	MAP in mm Hg	111	109	114	118	100	92	81	60
	Epi	+8	+6	+3	+10	+22	+17	+38	+50
	CO	+49	+35	+46	+42	+30	+21	+12	+10
	RCV	+26	+38	+39	+21	-9	+13	+8	+3
" , 1	MAP in mm Hg	105	100	93	91	92	80	75	68
	Epi	+31§	+30	+35	+67	+63	+74	+64	+68
	CO	+46	+16	+39	+16	+21	+23	+18	+15
	RCV	+9	-18	-18	-24	-16	-20	-21	-26

* Epinephrine, 3 µg/kg.

† Carotid occlusion.

‡ Right central vagal stimulation.

§ Change in mm Hg.

definite changes were observed in the responses to bilateral carotid occlusion and right central vagus stimulation as compared to control studies(9). However, 1 and 3 µg/kg of epinephrine evoked pressor responses which were generally greater, both in an absolute and relative sense than in tests before drug administration.

A delayed hypotensive response occurred 2½ to 3 hours following intravenous injection of both doses of pseudoreserpine and following the 1 mg/kg dose of raunescine (Table II). The 0.3 mg/kg dose of raunescine evoked no fall in MAP.

In all dogs there occurred an increase in pressor response to epinephrine administered intravenously. The pressor response to bilateral carotid occlusion was greatly reduced in both pseudoreserpine-treated dogs, but not after raunescine. There was a reduction or reversal of the primary pressor response following right central vagal stimulation in all dogs except the dog on 1 mg/kg of raunescine. No significant changes were observed following acetylcholine, histamine, isopropyl-norepinephrine, tilting or stimulation of the right peripheral vagus.

Discussion. Pseudoreserpine and raunescine differ from reserpine and deserpidine respectively by the presence of an hydroxyl rather than a methoxyl group at C-17 in ring

E. The substitution of a hydroxyl group for the methoxyl group reduced the potency of the compound to one-tenth in causing mice "fall-outs" from roller cages (Table III). Concerning MAP and HR in dogs, alterations in the C-17 group resulted in potency changes similar to those observed in mice, namely reduction of the bradycardic and hypotensive effects. Thus, a change in the C-17 group resulted in parallel potency changes in dogs and mice. Such parallelism does not necessarily follow when other changes in the molecule are effected, as shown by the relative potency of reserpine and rescinnamine. If a methoxyl group is retained at C-17 and the trimethoxybenzoyl group of reserpine is replaced by a trimethoxycinnamyl group, thus forming rescinnamine, potency of the compound as measured by "fall-outs" was reduced to one-thirteenth (Table III). On the other hand, when potency was measured by

TABLE III. Comparison of Effect of Alkaloids on Mice "Fall-Outs" from Roller Cages.

Alkaloid	ED ₅₀ * (mg/kg)	Slope function*
Raunescine	7 (5 -10)	5.0 (2.5-9.7)†
Pseudoreserpine	11 (8 -14)	2.7 (1.8-4.1)
Reserpine	0.8 (.62- 1.10)	2.1 (1.7-2.6)
Deserpidine	1.2 (.8 - 1.8)	2.2 (1.3-3.8)
Rescinnamine	8.6 (7 -10)	1.6 (1.3-2.0)

* With 95% confidence limits.

† Not parallel to the other lines.

degree of hypotension and bradycardia produced in dogs, rescinnamine was twice as potent as reserpine.

The results indicate that both alkaloids are less potent than reserpine, rescinnamine or deserpidine. Shore and Brodie(10) recently found that both pseudoreserpine and raunes-cine reduced the serotonin content of rabbit brains but were both significantly less potent than reserpine.

Summary and conclusions. Two new alkaloids from *Rauwolfia canescens*, pseudoreserpine and raunes-cine have been studied for pharmacologic activity in mice and dogs. Both pseudoreserpine and raunes-cine have reserpine-like activity, but are less potent than reserpine. The reduction in potency has been attributed to the substitution of the methoxyl group on C-17 in ring E by a hydroxyl group.

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Received June 7, 1957. P.S.E.B.M., 1957, v95.

Mode of Action of Aliphatic Amino Acids on Cortical Synaptic Activity. (23366)

DOMINICK P. PURPURA,* MARTIN GIRADO AND HARRY GRUNDFEST†

*Departments of Neurological Surgery and Neurology, College of Physicians and Surgeons,
Columbia University, N. Y. City*

Electrophysiological and pharmacological evidence has disclosed that hyperpolarizing, inhibitory synapses, developing surface-positive cortical postsynaptic potentials (p.s.p.'s) occur abundantly on the superficial dendrites in the cerebral cortex, but not in the cerebellar(1,2). Recognition of this difference has facilitated analysis of the modes of action of various pharmacological agents on central synapses(1-5) that are otherwise obscured in the character of the overt response(6,7).

* Scholar, Kenny Foundation. Supported in part by funds from Donner Fn. and Natl. Inst. of Neurological Diseases and Blindness.

† Supported in part by grants from Muscular Dystrophy Associations, Nat. Inst. Neurological Diseases and Blindness, Natl. Science Fn. and United Cerebral Palsy Research Fn.

Thus, central nervous system "stimulants" that produce convulsive activity by blocking inhibitory synapses (*e.g.* strychnine) may be distinguished(1,2) from those that activate excitatory synapses (*e.g.* Metrazol). The differential analysis is based on the absence of effect of topically applied strychnine-like agents on the cerebellar cortex, while topically applied activators of excitatory synapses produce convulsive activity in the cerebellar as well as the cerebral cortex. Differential analysis can also disclose the mode of action of "inhibitory" substances. If that overt effect results from selective blockade of excitatory synapses, topical application of the pharmacological agents to the cerebral cortex may be expected to produce inversion of the surface-negative dendritic p.s.p. to positivity as

the hyperpolarizing synaptic activity becomes unmasked. In the cerebellar cortex there will be only a disappearance of electrical response. This has been shown(3) to be the mode of synaptic action of topically applied γ -aminobutyric acid (GABA). The test just described does not detect directly another possible mode of inhibitory action, the selective activation of inhibitory synapses. However, this can also be analyzed by exclusion and by several indirect tests. Attention has recently been focused on the pharmacological role of GABA because this substance occurs in the brain in relatively high concentration(8,9) and because it has been identified as an active component of the "inhibitory" Factor I(10).[‡] However, other ω -amino acids also exert "inhibitory" or "excitatory" effects on electrical activity of dog motor cortex. The aliphatic acids with 4 carbons or less were found to be "inhibitory" and those with 5 or more were reported to be "excitatory"(14).

Because the overt manifestations do not necessarily indicate the precise type of synaptic action, the present experiments were designed to analyze the synaptic effects of a series of ω -amino acids. A comparative study is also included of the effects of some α -amino and α,ω -diamino acids. The work included examination of their actions on evoked dendritic p.s.p.'s of cerebral and cerebellar cortex, on the simultaneously recorded electrocortical activity, and in some instances also on the direct and synaptic excitability of cortico-spinal neurons. This report will be limited to the effects produced by topical applications of the compounds. Data obtained by

systemic or intraventricular injections will be described elsewhere.

Methods. The results included here are based on a series of 30 cats, all succinylcholine-paralyzed, unanesthetized preparations described earlier(1). Dendritic p.s.p.'s were evoked from the cerebral or cerebellar cortex by stimulating the cortical surface with a pair of fine (100 μ), closely spaced, teflon-insulated silver wires 0.5 to 2.0 mm distant from a stigmatic silver electrode recording from the cortex against an indifferent electrode on the frontal sinus. The responses were registered oscillographically, 2 traces of the cathode ray tube permitting simultaneous recording of independently evoked responses in different cortical regions. Bipolar or monopolar leads to a multi-channel ink writing oscillograph also recorded the "spontaneous" electrocortical activity of the same and other cortical areas. For examining the excitability of cortico-spinal neurons, one or a pair of teflon-insulated fine silver wires were inserted into the pyramidal tract to record oscillographically the descending activity resulting from stimulation of the motor cortex. In most experiments the tests were carried out in the following standardized procedure. The cortical regions under study were flushed with warm Ringer's solution and a series of 10 to 20 control responses, evoked every 2 sec. were recorded. Then 3 to 5 drops (0.1 to 0.2 ml) of the test amino acid solution (0.1 or 1.0% w/v, buffered to pH 7.4 with a synaptically inert buffer)[§] were applied to the testing cerebral and cerebellar areas. Since these were generally the anterior or middle suprasylvian gyrus and the posterior surface of the vermis, only a small fraction of the applied substance remained at the tested site. An approximate index of the amount remaining was obtained by comparing the quantity of GABA applied to the cortex on filter paper with the amount applied as the fluid required to produce approximately similar effects. Thirty μ g of GABA applied on a 4 mm² piece of the

[‡] Several lines of evidence strongly indicate that GABA is not the only active component of Factor I. Indeed, its central nervous action probably is very restricted. GABA affects only axo-dendritic, surface synapses and primarily by topical application(3). It does not protect mice against strychnine (unpublished work in this laboratory), while certain preparations of Factor I(11), although not others(12), are powerful antagonists of strychnine. Furthermore, that portion of brain extract which does not contain free amino acids has an "inhibitory" action on crayfish stretch receptors as powerful as that of GABA (cf. 10). A peripheral synaptic action of GBA is indicated, however, by its vasomotor effects in rabbit(13).

[§] Tris (hydroxy-methyl-amino-methane) does not affect electrical activity of the cortex. Other commercially available buffers, particularly those containing amino acids, were found to depress synaptic responses.

TABLE I. Cortical Synaptic Actions of Aliphatic Amino Acids.

Carbon No.	α -amino acids	ω -amino acids	α, ω -diamino acids
2	— — — — — Glycine — — — — —		×
3	0 (α -alanine)	— — — — — (β -alanine)	×
4	0 (α -aminobutyric)	— — — — — (γ -aminobutyric)	— (2, 4 diaminobutyric)
5	0 (Norvaline)	— — — — — (δ -amino <i>n</i> -valeric)	0 (Ornithine)
6	0 (Norleucine)	+ + + (ϵ -amino caproic)	+ (Lysine)
8	×	+ + + + (ω -amino caprylic)	×

Symbols: — to — — — — indicate increasing blockade of excitatory synapses which leads to overt "inhibitory" action. + to + + + + represent increasing blockade of inhibitory synapses leading to "excitatory" effects. 0, compound not active; ×, not available or not tried.

filter paper exerted about as much effect as 200 μ g in solution dropped on the cortex. The latter mode of application was used in the testing procedure because the effects were obtained rapidly (within 30-45 sec.) and were easily reversed by flushing the cortical surface with Ringer's solution. The maximum time of action of any substance was limited to 5 min. If another agent was to be tested at the same site, the application was made only after 20 to 30 min. during which time the cortex was repeatedly flushed with Ringer's solution.

Results. Six aliphatic amino acids (C_2 to C_6 and C_8), all with the amino group on the terminal carbon (ω -compounds), were found to have synaptic action (Table I). Whereas the α -amino isomers of the C_3 to C_6 ω -compounds were inert, 2 of the 3 α, ω -diamino acids (C_4 , C_6) also produced minimal synaptic effects.||

The C_2 to C_5 ω -amino acids all had similar actions, causing a change of the evoked cerebral dendritic p.s.p.'s from surface-negativity toward surface-positivity (Fig. 1). With the standard testing procedure it was often found that the C_2 (glycine) and C_5 (δ -amino *n*-valeric acid) compounds were not able to block the depolarizing component sufficiently to unmask the hyperpolarizing, surface-positive p.s.p. This difference, however, was merely quantitative, their action in the

range of concentrations used developing more slowly than did those of the C_3 and C_4 compounds. When applied in higher concentrations and for longer times, the less strongly acting C_2 and C_5 compounds reversed the surface potentials of evoked dendritic responses in the cerebral cortex. In the cerebellar cortex their action, like that of GABA(3), was the elimination of an evoked response. Although β -alanine (C_3) did not in general invert the cerebral dendritic p.s.p. as rapidly as did GABA, it nevertheless often produced a more profound surface positivity than did the C_4 substance.

The 2 longer chain ω -amino acids, C_6 (ϵ -amino caproic) and C_8 (ω -amino caprylic), produced entirely different effects from those of the C_2 to C_5 compounds. Both augmented the evoked dendritic p.s.p.'s of the cerebral cortex, but did not affect those of the cerebellum (Fig. 2); the differential test which indicates that the C_6 and C_8 ω -amino acids selectively block inhibitory synapses(1,2). A quantitative difference between the C_6 and C_8 compounds, as well as the qualitative difference between these 2 and the shorter chain ω -amino acids, are both strikingly shown by the relative effects on the cerebral electrocorticograms (Fig. 3), and on excitability of cortico-spinal neurons (Fig. 4).

The C_2 to C_5 compounds altered the electrocorticogram relatively little, their action—most pronounced in the case of GABA—be-

|| Other derivatives have also been tested, and the results will be reported elsewhere.

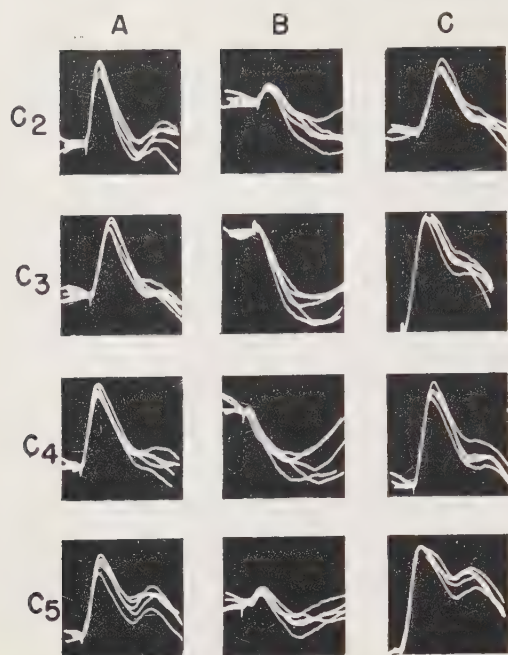


FIG. 1. Blockade of depolarizing, surface-negative dendritic p.s.p.'s in cat cerebral cortex by ω -amino acids. For illustrative purposes, all tests were performed on the same preparation. About 0.2 cc each of glycine (C_2), β -alanine (C_3), γ -aminobutyric acid (C_4) and δ -amino *n*-valeric acid (C_5) were applied to the cortex in 1% buffered solution at 20 to 30 min. intervals. A: Five superimposed consecutive records of the dendritic p.s.p. evoked immediately before applying the amino acid. B: 2 min. after the application. The C_2 and C_5 compounds produced less surface positivity than did the C_3 and C_4 ω -amino acids under the experimental conditions and a remnant of surface negativity is seen in both cases. C: 5 min. after flushing the cortex with warm Ringer's solution. The temporary augmentation of the dendritic surface-negativity after the C_3 to C_5 compounds was characteristic. Time, 20 msec.

ing to slow the rate and increase amplitude of the "spontaneous" activity (Fig. 3A to D). The C_6 compound, on the other hand, induced occasional paroxysmal discharges when applied for 3 to 5 min. (Fig. 3E). The C_8 ω -amino acid evoked violent paroxysmal discharges within 30 sec. after application of the 1% solution and this activity often progressed to sustained seizure discharges (Fig. 3F).

Neither the shorter chain amino acids which blockade selectively the excitatory dendritic synapses, nor the C_6 compound, which selectively blocks the inhibitory synapses, had any noticeable effect upon the excitability of cor-

tico-spinal neurons to direct or synaptic stimulation (Fig. 4; C_4 , C_6). The C_8 compound, on the other hand, progressively increased the indirect, relayed tract activity (Fig. 4; C_8), an action that is similar to the effect of strychnine, another strong inactivator of inhibitory synapses (15).

Several other results will only be mentioned briefly. Following washing out of the C_3 to C_5 ω -amino acids, there is "paradoxical" augmentation of the surface-negative dendritic p.s.p. of the cerebral cortex (Fig. 1C) above the control value (Fig. 1A). Decreased effectiveness of a given compound when it is repeatedly tested in a cycle of applications and washings is probably a tachyphylactic phenomenon. Several varieties of interaction between different compounds were also observed, but only one has been studied in detail. Although GABA itself blocks excitatory synapses its application to cortex already treated with C_6 or C_8 (Fig. 5) augments their convulsive action. This effect is increased by adding more of the latter compounds (Fig. 5C), but also by diluting both amino acids upon flushing the cortex with Ringer's solution (Fig. 5F).

Comments. The "inhibitory" and "excita-

CAPROIC (C_6)



CAPRYLIC (C_8)



FIG. 2. Differential actions of caproic (C_6) and caprylic (C_8) ω -amino acids on cerebral and cerebellar cortex. Records for the 2 compounds taken from 2 different experiments. Direct comparison of the augmentation produced by C_6 and C_8 on the evoked p.s.p. of cerebral cortex cannot be made. 1, 4, controls; 2, 5, one min. after application of agents to cerebral (upper trace) and cerebellar cortex; 3, 6, twenty min. after frequent rinsings of the cortical sites with warm Ringer's solution. The striking augmentation of responsiveness observed in cerebral cortex (2, 5) does not occur in the cerebellar cortex. Time, 20 msec.

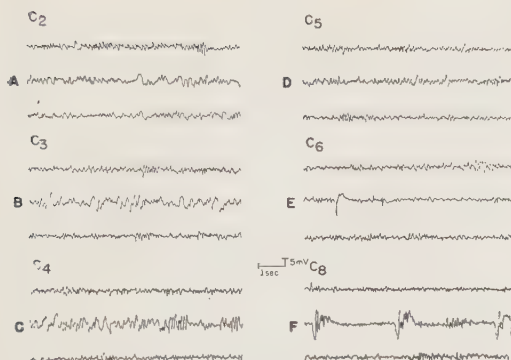


FIG. 3. Effects of topical applications of ω -amino acids on cerebral electrocorticograms. Same experiment as in Fig. 1. Bipolar leads, one on the anterior suprasylvian, the other on posterior sigmoid gyrus. In each set (A-F), a subscript (C_2 to C_8) identifies the amino acid tested. The upper record of the set was taken before applying the compound, the middle 2 min. after its application, and the lower 5 min. after flushing the cortex with warm Ringer's solution. Pronounced slowing and increased amplitude of the activity were induced maximally by the C_3 and C_4 ω -amino acids (B, C). The C_6 compound induced occasional hypersynchronous discharges (E), while the C_8 amino acid caused rhythmical high amplitude discharges and eventually, convulsive activity. In all cases the electrocortical actions were rapidly reversed.

tory" central nervous effects of the aliphatic ω -amino acids (14) were confirmed in the present work and their modes of synaptic action elucidated. Both types of effects are caused by inactivation (blockade) of synapses. The overt "inhibitory" result is caused by elimination of depolarizing, excitatory p.s.p.'s, and the compounds that produce this effect (the C_2 to C_5 substances) all act on both the cerebellar and the cerebral cortical responses, but in a different manner at the 2 structures. The "excitatory" action exerted by the C_6 and C_8 ω -amino acids is due to selective blockade of inhibitory synapses, and accordingly these compounds, like strychnine, do not produce cerebellar effects on topical application.

There is a quantitative aspect to the effects of the different ω -amino acids within each group. Among the shorter chain compounds, glycine (C_2) and δ -amino *n*-valeric acid (C_5) seem to have the weakest action as blockaders of depolarizing synapses. β -Alanine (C_3) and GABA (C_4) exert almost equally a much greater action. Of the 2 longer chain ω -amino acids tested, the C_8 compound has a much

more powerful selective action than does the C_6 compound, and on topical application ω -amino caprylic acid is as strongly acting as is strychnine. Like strychnine, the C_8 amino acid also affects the hyperpolarizing inhibitory synapses of the axo-somatic type. The paroxysmal action of both is associated with marked augmentation of the synaptically excited component of pyramidal tract activity after a single cortical stimulus (Fig. 4).

The various differences between the different length ω -amino acids presumably are ascribable to varieties of molecular configurations of synaptic membrane. The same factor probably also accounts for ineffectiveness of α -amino acids, and for loss of effectiveness when an α -amino group is added to an ω -amino acid (Table I). The fact that glycine, an α -compound, but with the amino group terminal, has synaptic action, like the longer

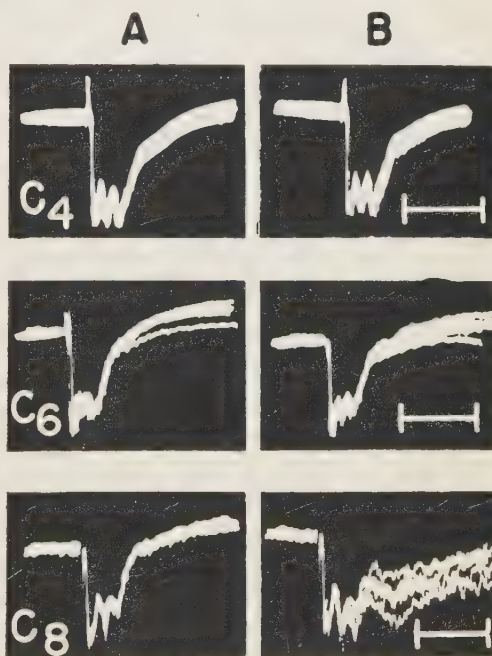


FIG. 4. Effects of C_4 , C_6 and C_8 ω -amino acids on evoked activity in pyramidal tract recorded at mid-olivary level following motor cortex stimulation. A, control; B, 10 min. after repeated application of the solutions (0.2 cc, 10^{-2} w/v in each case) to motor cortex. Each of the agents tested in a different experiment. Ten superimposed tracings for the C_4 , and 5 superimposed tracings for the C_6 and C_8 series. Significant augmentation in synaptically evoked late responses occurs only with C_8 ω -amino acid. Time, 10 msec.

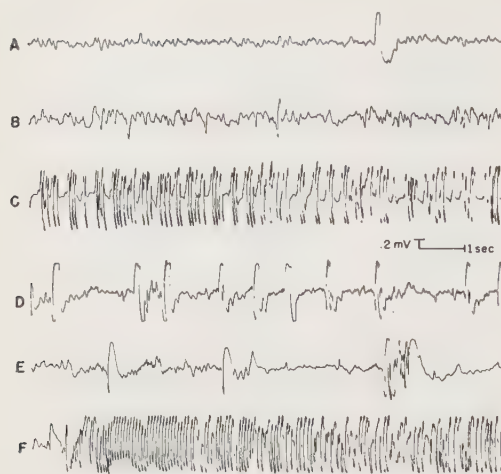


FIG. 5. Interactions of C_4 - C_6 (A-C) and C_1 - C_8 (D-F) ω -amino acids. A: Cerebral electrocorticogram after applying C_6 ; B: one min. after application of γ -aminobutyric acid; C: 30 sec. after application of additional C_6 . D-F: Another experiment. D, electrocorticogram after C_8 . E, one min. after γ -aminobutyric acid was added to C_8 . F, 30 sec. after flushing both agents from cortex.

chain ω -amino acids, suggests that the polar separation of the carboxy and amino groups is important to the pharmacological action. Other data also show that the carboxy group is necessary in the amino acid series, but several types of more complex structures are also synaptically active, as will be reported elsewhere. Adding, as they do, a new series of compounds of analyzable synaptic action and of readily modifiable structural complexity, the data described here therefore provide new means for study of molecular organization in synaptic membrane.

Conclusion. Several types of electrophysiological tests have been applied to the analysis of the pharmacological actions of amino acids. Only the ω -amino acids have been found to have pronounced synaptic effects among the available C_2 to C_8 compounds. The α -amino acids, with the exception of glycine which has

its amino group in the terminal position, are not synaptically active. Synaptic action is reduced or abolished by conversion to the α , ω -diamino compound. The ω -amino acids all act to blockade synapses, but quantitative and qualitative differences characterize the compounds of different carbon length. The C_2 to C_5 compounds block predominantly the depolarizing, excitatory synapses, while the C_6 and C_8 ω -amino acids block selectively the hyperpolarizing, inhibitory synapses. Within each group there are quantitative differences in effectiveness of selective action. The findings provide a new series of pharmacological tools for the analysis of the molecular structure and organization of central nervous synapses.

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Received June 13, 1957. P.S.E.B.M., 1957, v95.

Humoral Regulation of Erythropoiesis. IV. Relative Heat Stability of Erythropoietine. (23367)

FREDERICK STOHLMAN, JR. AND GEORGE BRECHER

Natl. Inst. of Arthritis and Metabolic Diseases, N.I.H., Bethesda, Md.

The heat stability of erythropoietine, a substance in the plasma of anemic animals capable of stimulating erythropoiesis, has been the subject of recent controversy. Borsook(1) originally reported that the plasma of rabbits with phenylhydrazine-induced anemia retained its effectiveness after deproteinization by boiling for 10 minutes at pH 5.5. Subsequently Gordon(2), Linman(3) and Mirand(4) reported similar observations. In Erslev's(5) experience as well as our own(6,7), however, plasma so treated was ineffective. In the latter instances plasma from animals with post-hemorrhagic anemia or those which had been exposed to altitude was used as the source of erythropoietine. Some recent studies have indicated that boiled plasma does not produce a uniform response(8,9,10). Jacobsen(8) first noted this in studies on plasma from animals with phenylhydrazine anemia. She suggested that the erythropoietic activity could be demonstrated in boiled plasma only if the phenylhydrazine intoxication had been severe enough to induce significant liver damage. Prentice and Mirand(11) noted that the plasma from hypoxic animals with liver damage due to carbon tetrachloride was more effective than that of hypoxic animals with normal livers. In many of the above studies the assay used for the demonstration of erythropoietic activity differed as did the method of production of erythropoietine in the donor animals. From the published data it was impossible to decide whether the discrepant reports were due to the use of different bio-assays, a relationship of activity to liver function, a multiplicity of humoral factors(12) or whether erythropoietine was indeed heat labile. The present study was undertaken in an effort to resolve these questions.

Methods and materials. Types of assay. Three different assay technics were utilized: (a) The sublethally irradiated animal previ-

ously reported by us(6). (b) The hypophysectomized animal suggested by Fried *et al.* (13) and Mirand and Prentice(4). Male Sprague-Dawley rats weighing 150 g were hypophysectomized* and used either 8-10 days after hypophysectomy as suggested by Fried(13) or 30-40 days as recommended by Mirand and Prentice(4). At the time of assay, the animals weighed from 120-160 g. They were randomly assigned to groups of 5-7 animals. The plasma to be tested was injected intravenously in amounts equivalent to 2% of the recipient's body weight. In one experiment a similar amount of boiled plasma was given subcutaneously daily for 3 days. Forty-eight hours after the initial injection of plasma, Fe^{59} bound to homologous plasma was administered intravenously. Forty-eight hours thereafter the amount of Fe^{59} incorporated into the circulating red cells was determined using a blood volume value of 6% of the body weight. (c) Normal animals were injected subcutaneously daily for 10-12 days with amounts of boiled plasma extract equivalent to 2% of the body weight. Five to 7 days prior to the institution of treatment 0.2-0.3 cc of blood was withdrawn by cardiac puncture for the determination of red cell values. At the time of the last injection of plasma, Fe^{59} tagged homologous plasma was given I.V. and 24 hours thereafter the Fe^{59} incorporation determined. At this time the red cell values were again determined. *Preparation of plasma.* Plasma from 4 sources was utilized; plasma from rats or rabbits made anemic by bleeding(6), plasma from normal rats exposed to a simulated altitude of 23,000 feet for 24 hours(14), plasma from rabbits treated with phenylhydrazine. In the latter instance rabbits weighing 1.5-2.5 kg were given 12.5 mg/kg of phenylhydrazine for 4-5 days and 48 hours after the last injection bled by cardiac puncture. At that time the hemato-

* Hormone Assay Laboratories, Chicago, Ill.

TABLE I. Effect of Temperature on Erythropoietine.

Control	Anemic plasma			
	Temperature of incubation			
	25° 24 hr	50° 6 hr	60° 0.5 hr	70° 0.5 hr
24 ± 5.2 (7)*	38 ± 5.7 (6)	40 ± 2.7 (4)		
23 ± 2.3 (6)			28 ± 3.2 (6)	27 ± 3.5 (4)
26 ± 3.4 (9)				

* Fe^{59} values given represent % of total inj. dose present in circulating red cells 48 hr after inj. $\pm$ a single stand. error. No. of animals in each group is included in parentheses. The sublethally irradiated rat was used for the assay.

crits ranged from 12-20 and reticulocytes from 60-95%. Liver damage was apparent grossly in about 25% of the animals so treated. Microscopic examination was in agreement with gross findings in the first 25 animals and thereafter was omitted. Boiled plasma extract was made as described by Borsook(11). The plasma was adjusted to pH 5.5 and then placed in a 100°C water bath for 10 minutes. The precipitate was separated by centrifugation. An amount of water equivalent to the original volume was added to the precipitate. This was agitated with a mechanical stirrer for 10 minutes and the wash water removed after centrifugation. This step was repeated a second time. The supernatant and wash water were pooled and either evaporated to the desired volume in a rotating evaporator at 50°C or lyophilized and the residue resuspended in ion free water. No differences were noted between the 2 methods of concentration. The pH was then adjusted to 7.0 with 1 N NaOH.

Results. The effectiveness in sublethally irradiated animals of altitude or anemic plasma was determined following incubation at 25°C for 24 hours, 50°C for 6 hours, and

60° or 70°C for 30 minutes. Following exposure at 60° or 70°C an erythropoietic effect could no longer be demonstrated (Table I). Boiled acidified plasma from rabbits with post-hemorrhagic or phenylhydrazine anemia was ineffective when tested in the sublethally irradiated animal. Since a number of investigators had described activity when multiple injections were given to normals, these fractions were tested according to the method described by Linman and Bethell(3). Again activity could not be demonstrated.

Concentrated fractions from homologous as well as heterologous plasma were then tested in both hypophysectomized and sublethally irradiated animals. A 3-fold concentration of boiled plasma was used so that each animal received the boiled extract of an amount of plasma equivalent to 6% of the recipient's body weight. By this increase in dosage significant stimulation of erythropoiesis by boiled anemic or altitude plasma was achieved in each of 4 experiments in which hypophysectomized animals were used (Table II). Stimulation of erythropoiesis was also noted in 4 experiments in the sublethally irradiated animal. However in both hypophysectomized

TABLE II. Effect of Boiling on Erythropoietine.

Source and treatment of plasma	Equivalent amount of native plasma (cc)	Fe^{59} incorporation	
		Irradiated rat assay	Hypophysectomized rat assay
None	—	16 ± 3.3 (7)*	40 ± 3.1 (8)
Normal rat	3	23 ± 7.3 (7)	40 ± 6.4 (7)
Altitude "	3	36 ± 3.7 (7)	65 ± 3.5 (6)
Boiled normal rat	9	21 ± 4.5 (6)	37 ± 2.2 (7)
" altitude "	9	26 ± 2.3 (6)	52 ± 1.69 (7)
" normal rabbit	9	22 ± 2.8 (6)	43 ± 5.5 (6)
" phenyl " with liver damage	9	27 ± 2.9 (6)	55 ± 2.2 (7)
<i>Idem</i> , without liver damage	9	32 ± 3.6 (7)	69 ± 2.3 (7)

* Fe^{59} values given represent % of total inj. dose present in circulating red cells 48 hr after inj. $\pm$ a single stand. error. No. of animals in each group is included in parentheses.

TABLE III. Effect of Dilution on Erythropoietic Activity of "Altitude" Plasma.

Source of plasma	Amt of plasma given (% of body wt)	% Fe ⁵⁹ incorporation
Normal plasma	2 %	20 ± 3.3 (6)*
Altitude "	2	35 ± 3.0 (6)
" "	1	32 ± 2.8 (6)
" "	0.5	26 ± 2.0 (6)

* Fe⁵⁹ values given represent % of total inj. dose present in circulating red cells 48 hr after inj. ± a single stand. error. No. of animals in each group is included in parentheses. The sublethally irradiated rat was used for the assay.

and irradiated animals unconcentrated native altitude plasma produced a greater response ($p < .05$)[†] than did 3 times the amount of boiled acidified altitude plasma obtained from the same plasma pool. This indicated that more than 70% of the erythropoietic activity had been destroyed on heating. In an effort to quantify further the erythropoietic response a dilution experiment utilizing fresh plasma was conducted (Table III). Here, an amount of plasma equivalent to 0.5% of the body weight gave a response similar to that observed from the extract of 12 times as much boiled plasma. It is not suggested that these are quantitative determinations but merely rough semi-quantitative measures indicating that between 70-90% of the activity is lost on boiling for 10 minutes.

In the experiment presented in Table II the plasma derived from phenylhydrazine treated rabbits with normal livers gave a greater response than did that from animals with liver damage. In the hypophysectomized assay this difference was significant ($p < .02$). In 2 other experiments significant stimulation of erythropoiesis was produced by

the injection of concentrated boiled extracts of phenylhydrazine plasma obtained from rabbits with normal livers.

Finally, the stability of acidified phenylhydrazine plasma was determined after prolonged periods of boiling (Table IV). Activity could be demonstrated in unconcentrated plasma which had been boiled for 5 minutes but not after boiling for 30 or 60 minutes (Table IV). It may be recalled that in previous experiments activity could no longer be demonstrated in unconcentrated plasma boiled for 10 minutes. Concentration, however, permitted detection of activity in those aliquots boiled for 30 or 60 minutes, as had been the case previously with plasma boiled for 10-15 minutes. The response to concentrated plasma boiled for 30 minutes was less than that of plasma boiled for 5 minutes, though not significantly. No further decrease was noted after boiling for 60 minutes.

Discussion. These data demonstrate that erythropoietic activity is greatly decreased but not completely destroyed by prolonged boiling. While the assay technics used are semiquantitative it nevertheless was possible to estimate that 70-90% of the activity was destroyed by heating acidified plasma at 100° for 10 minutes. Heating for periods of 30-60 minutes did not produce a further decrease in activity. Thus, it is evident that the reaction is non-linear and likely that the kinetics of this reaction are complex. The assay technic is undoubtedly too insensitive to detect differences of a few percent which might occur at the tail of a simple exponential curve or a higher order reaction. Another possibility is that a portion of the circulating erythropoie-

TABLE IV. Effect of Duration of Boiling on Erythropoietine Activity.

	Control	Normal rabbit plasma, boiled 5 min.	Phenylhydrazine rabbit plasma		
			Boiled for		
			5 min.	30 min.	60 min.
Unconcentrated	19 ± 1.44 (6)*	10.8 ± 2.7 (6)	27 ± 2.9 (6)	20 ± 3.1 (6)	20 ± 2.0 (6)
3-fold conc.	19 ± 4.6 (6)	22 ± 3.2 (6)	36 ± 2.8 (6)	32 ± 5 (6)	32 ± 2.9 (6)

* Fe⁵⁹ values given represent % of total inj. dose present in circulating red cells 48 hr after inj. ± a single stand. error. No. of animals in each group is included in parentheses. The sublethally irradiated rat was used for the assay.

[†] Significance was determined using a one-sided t test.

tine is protein bound and that the bound form is heat stable. The data do not exclude the existence of 2 humoral factors, one heat stable and the other labile(12). Since the observations do not necessitate the assumption of a second heat stable erythropoietine, however, it would seem justifiable for the present to consider that there is a single humoral factor a major portion of which is destroyed by boiling.

These experiments are in concert with the reports of Mirand and Prentice(4) who were able to detect erythropoietic activity in all instances where concentrated boiled plasma extracts were utilized. Recently Erslev(15) has also been able to demonstrate activity in boiled plasma when concentrates were used. The experience of these investigators as well as our own suggest that the inability to demonstrate activity in some lots of anemic plasma whether from human or animal sources is not necessarily due to the lack of erythropoietine in such plasma but may be due to the destruction of a large portion of the material prior to testing.

While no systematic study has been conducted on the role of the liver in relation to the level of erythropoietine, it is clear from these data that the presence of liver damage is not a prerequisite for the demonstration of erythropoietine in phenylhydrazine induced anemia (Table III). Erythropoietic activity was demonstrated on 3 occasions in a concentrated extract of acidified boiled plasma from rabbits with phenylhydrazine-induced anemia but with normal livers. It is possible that in a more extensive study, some correlation between the concentration of erythropoietine and the state of liver function could be established.

Summary and conclusions. Stimulation of erythropoiesis in the sublethally irradiated or hypophysectomized rats was demonstrable following injection of a 3-fold concentrated

acidified boiled extract of plasma from either rats exposed to simulated altitude or rabbits with phenylhydrazine-induced anemia. No relationship could be established between activity and coexisting liver disease in phenylhydrazine-treated animals. It was estimated that 70-90% of the erythropoietic activity was destroyed by heating acidified plasma at 100° for 10 minutes. Heating for 60 minutes did not further reduce activity. It is suggested that the small fraction of residual activity present after prolonged boiling does not warrant the designation of erythropoietine as a heat stable material nor does it necessitate the assumption of a separate heat stable component, although it does not entirely exclude this possibility.

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Received June 13, 1957. P.S.E.B.M., 1957, v95.

Diastolic Hypertension Produced by High Fat Diets and Dietary Stresses.* (23368)

C. M. WILHELMJ, V. W. MEYERS, AND H. H. MCCARTHY
(With the technical assistance of E. L. Freeland)

*Departments of Physiology-Pharmacology and Surgery, The Creighton University School of
Medicine, Omaha, Neb.*

Four normal healthy dogs that were subjected to high fat diets and severe dietary stresses and irregularities over a prolonged period of time gradually developed a highly significant diastolic hypertension of long duration. Previous publications have described by graphic illustration, the first circulatory abnormality to appear(1) and the genesis and development of the hypertension(2). It is the purpose of the present communication to present a statistical evaluation of the fully developed hypertension.

Methods and procedure. The 4 dogs (2 males and 2 females) had been used in blood pressure studies for approximately 5 years. During that time blood pressure had been determined 5 to 6 days each week with almost no interruption, consequently, the animals were well trained and standardized. They were mature adult animals of approximately the same age and the 2 females were still having regular estrous cycles.

Blood pressure was determined by the auscultatory method of Allen(3) with certain modifications(4,5). During the present studies, blood pressure and heart rate were determined by the same observer, 5 to 6 days weekly between 6 and 10 a.m., with a specific time for each dog. The daily blood pressure values were the mean of from 10 to 15 consecutive readings.

The normal kennel diet consisted of a weighed amount of Nutrena dog food which kept each animal at an optimal body weight and the amount fed was the same during the control and the hypertensive periods. During all phases of the study the animals were fed at 3:00 p.m.

The first period of dietary stress (Experi-

mental Period I) consisted of the following dietary manipulations done consecutively in the order given:

(A) A long preliminary fast (29 to 33 days) followed by realimentation with diets containing 50% of the calories from equal amounts of beef suet and salt free butter and 50% from cracker meal or horse meat, usually the former. These diets were fed at the level of 120 cal/M²/hr. The preliminary fast and realimentation with the high fat diets will be referred to as "fat episodes." In Experimental Period I these were repeated 6 times in 14 months. There was little or no gain in weight on the high fat diets.

(B) A diet containing one-half cracker meal and one-half raw horse meat fed at the level of 120 cal/M²/hr for 3 months. This diet caused very marked obesity. (C) The normal kennel diet for 3 months during which time weight loss was minimal. (D) A very low calory diet of raw horse meat for 5½ months which caused marked weight loss. (E) The normal kennel diet for 2 months. (F) The same low calory diet of raw horse meat for 2 months. (G) The usual kennel diet for 1½ months. (H) A diet of raw horse meat fed at the level of 120 cal/M²/hr for 39 days. This diet caused a rapid gain in weight. (I) The normal kennel diet for 3 months. This period terminated approximately 22 months after the end of the last fat episode. The blood pressure and heart rate during the final 3 months on kennel diet are shown in the Table as Experimental Period I.

The second period of dietary stress consisted of 3 "fat episodes" in 10 months. The duration of the preliminary fasts varied from 20 to 36 days. These differed from those in the first period of dietary stress in that the realimentary diet contained 70% of the calories from animal fat and in each "fat episode" the high fat diets were alternated ir-

* Financed by grants from The Nat. Heart Inst., The American Heart Assn. and The Nebraska Heart Assn.

regularly between the levels of 60 cal/M²/hr and 120 cal/M²/hr. Each fat episode was followed by a period on the low calory meat diet. At the end of this second period of dietary stress the animals were placed on the normal kennel diet for 84 days. The blood pressure and heart rate during the final 84 days on kennel diet are shown in the table as Experimental Period II.

The theoretical reasons for the use of the experimental design described above have been discussed(1,6).

(A) *Control blood pressure.* These 4 animals had been used in studies of the effect of protein and carbohydrate on blood pressure and heart rate(6). The control blood pressure and heart rate on the normal kennel diet were determined several times before, during and after these previous studies. It was found that protein and carbohydrate caused no permanent circulatory abnormalities and no change in the control blood pressure values on the kennel diet(7). The last control period was just before starting the present studies on animal fat. The number of control days on which blood pressure was determined varied from 89 to 168 for the 4 dogs. Since the value for each control day was the mean of from 10 to 15 readings, the number of determinations is large. The individual daily control values were combined statistically for each dog to give a single mean and standard deviation and from these the coefficients of variation and standard errors of the control mean value were calculated.

(B) *Blood pressure and heart rate after first period of dietary stress (Experimental Period I).* In Dogs I, III and IV elevations of the systolic and diastolic pressures above the control values were of about the same order of magnitude and were sufficiently great to be considered physiologically significant. Statistically the probability of these elevations calculated from *t* was <0.01. In Dogs I and IV the pulse rates were significantly lower than the control value but significantly higher than the control in Dog III.

In Dog II the actual elevation of the pressure above the control value was too small to be considered physiologically significant but the high statistical significance suggests that

the basic trend in this animal was the same as in the others. The pulse rate in this dog is significantly lower than the control value. The small number of readings made on this dog was due to the fact that while the experiment was in progress, before the statistical study was made, we considered this animal to be refractory to the dietary stresses and temporarily discontinued the daily determinations of blood pressure.

(C) *Blood pressure and heart rate after second period of dietary stress (Experimental Period II).* In Dogs I, III and IV the systolic and diastolic pressures were again significantly above the control values. The heart rate in Dogs I and IV was again significantly below the control values but was again higher in Dog III.

In Dog II the systolic and diastolic pressures became markedly elevated above the control values while pulse rate was significantly slower.

Discussion. Throughout the entire experimental period the various dietary changes were always made simultaneously on the 4 dogs so that the dietary stresses were of the same duration and intensity. In view of this it is interesting to note the tendency to individual susceptibility of the animals. The first experimental period resulted in a near maximal elevation of blood pressure in Dogs I and III, while Dog II showed little effect. The second experimental period caused a marked rise in the pressure of Dog II and additional elevation in Dog IV.

During Experimental Period I the stress of the "fat episodes" caused a number of complications such as jaundice, vomiting, anorexia, convulsions, tarry stools and high retention of bromsulphthalein. During the second experimental period, the "fat episodes" were well tolerated and the only abnormal sign noted was occasional tarry stools.

During Experimental Period I the first circulatory abnormality to appear was failure of the blood pressure to decline during fasting; it either rose and plateaued at high levels or more frequently rose progressively during fasts of several weeks' duration. This abnormality appeared gradually and was not fully developed in all dogs until after the

TABLE I. Effect of 2 Periods of Dietary Stresses on Blood Pressure and Heart Rate of 4 Normal Dogs.

Dog	Control on kennel diet				Exp. period I				Exp. period II				Difference from control and significance of difference							
	S		D		S		D		S		D		S		D		S		D	
	M	S.D.	M	S.D.	M	S.D.	M	S.D.	M	S.D.	M	S.D.	M	S.D.	M	S.D.	M	S.D.	M	S.D.
I	114	13.21	44	9.42	82	14.64	141	6.81	76	5.49	62	9.18	133	7.09	79	5.00	56	5.39	20	19
	115	11.5	21.4	17.8	17.8	1.31	4.8	.87	7.2	14.8	1.18	1.00	5.3	6.3	9.6	5.39	5.39	5.39	5.39	5.39
	118	.84	124	124	124	1.31	1.31	.87	.70	60	60	60	1.18	1.00	.70	.76	.76	.76	.76	.76
	124	124	124	124	124	1.31	1.31	.87	.70	60	60	60	1.18	1.00	.70	.76	.76	.76	.76	.76
II	130	14.7	61	12.50	68	11.36	140	5.70	67	3.16	55	1.22	166	20.91	102	21.97	51	5.33	13	36
	113	11.3	20.4	16.7	16.7	1.06	4.07	2.55	4.7	2.2	2.2	2.2	12.5	12.5	21.5	21.5	10.4	10.4	10.4	10.4
	113	113	113	113	113	1.06	1.06	2.55	1.41	5.4	5.4	5.4	5.4	5.4	3.13	.76	.76	.76	.76	.76
	113	113	113	113	113	1.06	1.06	2.55	1.41	5.4	5.4	5.4	5.4	5.4	3.13	.76	.76	.76	.76	.76
III	104	6.71	60	7.04	73	13.8	144	11.89	104	9.49	87	10.14	133	10.38	100	9.79	82	9.56	44	40
	6.4	11.7	11.7	18.9	18.9	1.46	8.3	9.1	9.1	11.6	11.6	11.6	7.8	7.8	9.7	9.7	11.6	11.6	11.6	11.6
	.71	.74	.74	1.46	1.46	1.46	1.54	1.23	1.23	1.32	1.32	1.32	1.48	1.48	1.39	1.39	1.36	1.36	1.36	1.36
	89	89	89	89	89	1.46	1.46	1.54	1.23	1.32	1.32	1.32	1.48	1.48	1.39	1.39	1.36	1.36	1.36	1.36
IV	114	10.40	59	9.15	79	10.86	143	10.22	96	10.83	67	6.92	153	15.45	105	12.79	71	9.91	12	39
	9.1	15.5	15.5	13.7	13.7	.83	7.1	11.6	11.6	10.3	10.3	10.3	10.0	10.0	12.1	13.9	13.9	13.9	13.9	13.9
	.80	.70	.70	.83	.83	.83	1.32	1.39	1.39	.89	.89	.89	2.20	2.20	1.82	1.45	1.45	1.45	1.45	1.45
	168	168	168	168	168	.83	1.32	1.39	1.39	.89	.89	.89	2.20	2.20	1.82	1.45	1.45	1.45	1.45	1.45

S, D, and P = Systolic and diastolic pressure and pulse rate.

M = Mean; S.D. = Stand. dev. of mean; C.V. = Coefficient of variation; S.E. = Stand. error of mean; N = No. of days on which blood pressure and heart rate were determined.

Identifying dog numbers are the same as in references 1 and 2.

sixth "fat episode." At this time the blood pressure would return to and remain at the control level for 51 days or more, if the animals were placed on the normal non-stress kennel diet; however, the abnormality was latent and would become overt as soon as fasting was started(1).

The above findings led to the hypothesis that the homeostatic mechanisms which tend to oppose the fall in blood pressure during caloric restriction had become hypereffective. If this hypothesis was correct it appeared that a prolonged period on a very low caloric intake might strengthen these homeostatic mechanisms and cause them to become permanently over-active even when the animals were on the non-stress, calorically sufficient kennel diet, and thus result in hypertension as a disease of adaptation as defined by Selye (8).

This hypothesis was tested experimentally and resulted in a significant hypertension while on the usual kennel diet. The condition now resembles benign essential hypertension since there is no retinopathy, albuminuria or azotemia.

The question may be raised as to whether the elevation of blood pressure of these 4 dogs may not have been spontaneous, due to ageing. This is obviously rather difficult to answer in a prolonged experiment of this kind, but the following points argue against this explanation: (1) The high pressure on the kennel diet became evident rather promptly after Experimental Period I in Dogs I, III and IV and after Experimental Period II in Dog II. (2) Two trained standardized dogs of approximately the same age as the 4 experimental dogs have been used in other phases of our blood pressure studies for a period of time approximately one year less than the 4 dogs reported here and have shown no spontaneous rise in blood pressure on the kennel diet. (3) Blood pressure of 2 other dogs of approximately the same age was previously found to be practically unaltered when on the kennel diet, after 4 years of observation.

It is provocative to note that these experiments unintentionally resemble the dietary habits of many inhabitants of countries where

hypertension is common, *i.e.*, over-indulgence in high fat-high calory diets resulting in obesity and followed by strenuous and irregular attempts to reduce—failure of the will power to continue the reducing regimen and a renewal of the cycle.

Summary. Four normal adult dogs were subjected to rather intense dietary stresses and irregularities over a prolonged period of time. Basically these dietary manipulations consisted of the following: (1) A prolonged preliminary fast followed by realimentation with diets containing 50 to 70% of the calories from beef suet and butter and fed at a level of from 60 to 120 cal/M²/hr. These "fat episodes" were repeated 6 times in 14 months in the first experimental period and 3 times in 10 months during the second experimental period. (2) The production of marked obesity by a high calory diet. (3) A long period on a low calory diet of raw horse meat which caused marked weight loss. (4) Return to the normal kennel diet. As a result of these dietary manipulations, the animals gradually developed a highly significant diastolic hypertension which is still present while on the normal kennel diet approximately 34 months after the end of the last fat episode of Experimental Period I.

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Received June 19, 1957. P.S.E.B.M., 1957, v95.

Effect of Ethinyl Estradiol on Plasma 17-Hydroxycorticosteroids, ACTH Responsiveness and Hydrocortisone Clearance in Man.* (23369)

ELEANOR Z. WALLACE, HERBERT I. SILVERBERG, AND ANNE C. CARTER

(With the technical assistance of Lydia S. Hersh.) (Introduced by David M. Kydd)

Department of Medicine, State University of New York, Brooklyn, and Medical Service

(Division II) Kings County Hospital.

Studies of the effect of estrogens on adrenal size and function in the experimental animal have yielded conflicting results. Administration of natural and synthetic estrogenic preparations has been followed by pituitary and adrenal hypertrophy in the intact rat(1,2,3), a phenomenon not seen in the hypophysectomized animal. In male rats, Gemzell(4) has reported an increase in plasma ACTH after administration of estradiol monobenzoate but Vogt(5) found that hexoestrol appeared to lower concentration of corticosterone in adrenal vein blood. Recently, Carter(6) was unable to demonstrate significant increases in adrenal weight in rats treated with estradiol dipionate.

In guinea pigs, Zondek and Burstein(7) have reported marked increases in urinary corticosteroid excretion during estrone and stilbesterol treatment. Chesterman, Franks, Knudsen and Williams(8) have recently described a picture highly suggestive of primary aldosteronism following long term stilbesterol therapy in the same species. In the rabbit, estrogens appear to have no effect on adrenal histology or corticosterone secretion(5).

Little is known in man of the effect of estrogens on adrenal secretory activity. In one study(9), although estradiol caused a decrease in total neutral 17-ketosteroids in the urine of male subjects, increases in daily excretion of dehydroisoandrosterone suggested an augmented adrenocortical secretion. More recently, Taliaferro, Cobey and Leone(10) reported elevated levels of plasma 17-hydroxycorticosteroids in patients with carcinoma of the prostate who were receiving large doses of stilbesterol. Such a rise did not

occur in 1 male patient with Addison's disease.

The present study was undertaken to evaluate adrenocortical function in castrate and post-menopausal females before and after administration of ethinyl estradiol.

Materials and methods. The subjects included 17 females who had had a spontaneous menopause 10 or more years before, 1 female 4 months post-surgical castration, 3 female patients with documented hypopituitarism due either to pituitary tumor or necrosis and a male with established Addison's disease. These patients received either 0.1 or 0.5 mg ethinyl estradiol in one oral daily dose.

Plasma 17 - hydroxycorticosteroids (17 - OHCS) were determined using the method of Silber and Porter as modified by Peterson (11). Normal control levels range from 4-30 $\mu\text{g}/100\text{ ml}$ of plasma in this laboratory.

The adrenocortical response to ACTH was measured by the effect of 25 U.S.P. units of ACTH in 500 ml 5% glucose in water given intravenously over 6 hours on plasma 17-OHCS levels. Control samples were obtained and infusions begun between 8 a.m. and 9 a.m.

Plasma hydrocortisone clearances were studied by administering 100 mg of hydrocortisone sodium succinate in one rapid intravenous injection and determining plasma 17-OHCS levels before and at 4, 6, and 8 hours following injection. Hydrocortisone administered in this fashion is cleared from the plasma of normal subjects within 6 hours(12).

Plasma samples were analyzed before and at 2, 4, and 6 hours after the ingestion of a single dose of 0.5 mg of ethinyl estradiol by mouth in 4 patients in order to determine whether this compound could cause a direct or artifactual elevation of plasma 17-OHCS.

Results. I. *Effect of ethinyl estradiol on plasma 17-hydroxycorticosteroids and on*

* This work was supported in part by a grant from the Upjohn Co., Kalamazoo, Mich. Ethinyl Estradiol (Estinyl) was generously supplied for this study by the Schering Corp. Corticotropin (Res. No. 10,918-3) was kindly supplied by the Upjohn Co.

TABLE I. Effect of Ethinyl Estradiol on Plasma 17-Hydroxycorticosteroids and on Their Response to ACTH.

Patient	Age	Description	Pre-ethinyl estradiol Plasma 17-OHCS, $\mu\text{g}/100\text{ ml}$		mg per day p.o.	No. of days	Post-ethinyl estradiol Plasma 17-OHCS, $\mu\text{g}/100\text{ ml}$	
			Pre-ACTH	Post-ACTH			Pre-ACTH	Post-ACTH
N.K.	61	10 yr post-menopause	12.4	35.6	.5	21	62.2	116.5
J.W.	66	10 " "	11.1	38.6	.5	21	94.4	156.2
L.F.	63	20 " "	20.4	48.4	.5	21	73.3	174.4
M.P.	85	30 " "	11.8	40.5	.5	21	79.3	116.7
A.G.	78	30 " "	19.6	43.5	.5	16	43.6	64.5
E.S.	74	30 " "	14.7	35.5	.5	16	64.7	103.1
R.H.	78	30 " "	22.8	48.3	.5	16	58.4	113.2
A.R.	65	16 " "	12.9	52.2	.5	16	50.6	102.3
M.P. ₁	64	20 " "	10.6	39.3	.5	16	57.4	113.7
M.P. ₂	76	30 " "	30.0	52.1	.1	11	62.5	—
M.J.	73	20 " "	20.2	—	.1	10	40.9	—
H.C.	75	25 " "	20.3	41.2	.1	17	54.3	89.7
J.N.	23	Pituitary tumor	3.8	12.1	.5	10	4.7	13.3
						30	5.5	—
C.G.	31	Post-partum pituitary necrosis	3.8	27.3	.5	12	18.1	64.2
						33	22.9	—
G.G.	36	17 yr postop. cranio- pharyngioma	8.4	30.0	.1	24	16.6	—
			2.0	—				
W.H.	37	Addison's disease	7.4	6.2	.5	14	6.1	—
						30	2.6	—

adrenocortical response to a standard ACTH test (Table I). Marked increases in concentration of plasma 17-hydroxycorticosteroids (17-OHCS) were seen in all post-menopausal women receiving ethinyl estradiol. Two of 3 patients with hypopituitarism (C.G. and G.G.) also showed definite increases in levels of plasma 17-OHCS. C.G., a 31-year-old female with post-partum pituitary necrosis, had normal thyroid function (RAI uptake 45% in 24 hours, PBI 9 μg %) and slightly decreased adrenocortical responsiveness (Table I) at the onset of the study. G.G., a 36-year-old female who had had a craniopharyngioma resected 17 years ago, had depressed thyroid function (RAI uptake 2.5% in 24 hours, PBI 2.4 μg %) and moderately impaired adrenocortical secretory capacity (Table I). The third patient with hypopituitarism (J.N.) did not have a rise in plasma 17-OHCS at the end of 30 days of ethinyl estradiol. This patient, a 23-year-old female with x-ray evidence of a pituitary tumor, had low normal thyroid function (RAI uptake 23% in 24 hours, PBI 3.6 μg %) and only a minimal response of urinary and plasma 17-OHCS to ACTH stimulation before administration of ethinyl estradiol (Table I).

A male subject with Addison's disease (W.H.) showed no rise in plasma 17-OHCS after ingestion of 0.5 mg of ethinyl estradiol for 30 days.

Ten post-menopausal patients with normal initial responses to corticotropin (plasma 17-OHCS after stimulation 36-52 μg %) had ACTH tests performed after plasma 17-OHCS levels were elevated during ethinyl estradiol therapy. At this time concentration of 17-OHCS in plasma rose to between 64-174 μg % at the end of the corticotropin infusion.

Two of the patients with hypopituitarism received corticotropin while taking ethinyl estradiol. Patient J.N. whose initial ACTH test revealed markedly depressed adrenocortical responsiveness showed no essential change when the test was repeated after 10 days of ethinyl estradiol. Patient C.G. with only moderate initial depression of adrenocortical function had a distinctly increased response of plasma 17-hydroxycorticosteroids to ACTH on the 12th day of ethinyl estradiol. Neither patient received any other medication during the study.

II. *Effect of ethinyl estradiol administration on plasma clearance of exogenous hydrocortisone* (Table II). Clearance of adminis-

TABLE II. Plasma Clearance of Intravenous Hydrocortisone.

Patient	Age	Description	Ethinyl estradiol mg per day p.o.	No. of days	Plasma 17-hydroxycorticosteroids, $\mu\text{g}/100\text{ ml}$			
					Control	4 hr	6 hr	8 hr
C.H.	75	20 yr post-menopause	.5	17	48.8	—	141.6	107.8
J.N.	23	Pituitary tumor	.5	30	5.5	140.0	123.3	—
C.G.	31	Post-partum pituitary necrosis	.5	33	22.9	194.6	149.6	—
G.G.	36	Craniopharyngioma, 17 yr post-op.	.1	24	16.6	99.8	77.6	—
D.K.	67	20 yr post-menopause	0		23.8	48.0	32.5	—
A.B.	63	11 " "	0		16.0	39.4	24.4	—

tered hydrocortisone from plasma has been evaluated in 4 patients: one 75-year-old woman who was receiving 0.5 mg ethinyl estradiol each day and the 3 patients who had hypopituitarism. Two of the latter were taking 0.5 mg ethinyl estradiol daily and the other 0.1 mg. In all 4 patients, there was evidence of a marked delay in clearance of the administered dose of Compound F. Two clearance studies in normal post-menopausal patients not receiving estrogens are presented in Table II for comparison.

III. *Effect of ethinyl estradiol on the Porter-Silber Color Reaction.* Administration of a single dose of 0.5 mg of ethinyl estradiol by mouth, failed to produce any increase in Porter-Silber chromogens at 2, 4, and 6 hours after ingestion (Table III). In all instances, results compatible with a normal diurnal variation of plasma 17-OHCS were obtained.

Crystalline ethinyl estradiol, 160 μg gave no absorption at 410 $m\mu$ when carried through the procedure for estimation of plasma 17-OHCS. This amount exceeds the concentration which could be achieved in the plasma of patients receiving either 0.1 or 0.5 mg of the drug daily.

Discussion. The data indicate that ethinyl estradiol produced elevations in plasma 17-

hydroxycorticosteroids in patients with adrenal cortices capable of normal or nearly normal function. Elevated levels were accompanied by an apparent augmentation of responsiveness of the plasma 17-OHCS to a standard infusion of 25 U.S.P. units of ACTH. Both effects were seen in all the post-menopausal females treated with ethinyl estradiol at either of the dose levels used in the study.

Although no rise in plasma 17-hydroxycorticosteroids was obtained in the patient with Addison's disease or in the patient with severe secondary adrenal cortical insufficiency associated with a pituitary tumor, distinct rises were encountered in the 2 other patients with hypopituitarism. Both of these latter patients had only moderately decreased adrenocortical secretory capacity at the onset of the study.

A distinct delay in rate of clearance of exogenous hydrocortisone was found in all 4 patients so tested while receiving ethinyl estradiol. Delayed plasma clearances of infused hydrocortisone have been reported previously only in patients with liver disease(12,13) and with myxedema(13), while normal rates of disappearance of hydrocortisone have been found in both primary and secondary adrenocortical insufficiency(11). Since only one of our patients had any significant degree of hypothyroidism (G.G.), the delayed clearances

TABLE III. Effect of a Single Dose of 0.5 mg Ethinyl Estradiol, p.o., on Plasma 17-Hydroxycorticosteroids.

Patient	Age	Description	Plasma 17-OHCS, $\mu\text{g}/100\text{ ml}$			
			Control	2 hr	4 hr	6 hr
J.M.	91	40 yr post-menopause	33.0	—	27.0	18.4
C.G.	31	Post-partum pituitary necrosis	9.0	3.7	3.0	.6
R.P.	75	25 yr post-menopause	32.9	28.2	19.1	23.8
I.C.	35	4 mo post-total panhysterectomy	20.1	8.9	6.6	8.1

uniformly encountered appear to be related to an alteration in hepatic handling of hydrocortisone in patients treated with ethinyl estradiol.

Hydrocortisone is normally metabolized in large part by a series of steps involving (1) removal from the circulation by the liver, (2) enzymatic reduction to its dihydro and then (3) tetrahydro derivatives, (4) conjugation with glucuronic acid and, finally, (5) reintroduction of the conjugated derivatives into the circulation for excretion by the kidneys. The delay in clearance of infused hydrocortisone from plasma found in this study could reflect alterations in steps 1-4 of this mechanism. The data at hand do not permit more exact localization of the effect of ethinyl estradiol in the liver. Studies are in progress in this laboratory to define more precisely the physiological alteration in hydrocortisone metabolism induced by ethinyl estradiol.

Summary. 1. Ethinyl estradiol in doses of 0.1 and 0.5 mg daily by mouth caused marked elevations in plasma 17-hydroxycorticosteroid levels in 18 post-menopausal and castrate females and in 2 patients whose hypopituitarism was associated with only moderate decreases in adrenocortical secretory capacity. 2. No increases in plasma 17-OHCS levels during ethinyl estradiol therapy were found in a male Addisonian and in a patient with a pituitary tumor and marked secondary adrenocortical insufficiency. 3. Elevations in plasma 17-OHCS were accompanied by augmented

responsiveness to a standard ACTH infusion in all 10 of the post-menopausal females studied and in 1 of 2 patients with hypopituitarism. 4. A marked delay in rate of plasma clearance of infused hydrocortisone was present in all 4 of the ethinyl estradiol treated patients so tested. 5. The data suggest that ethinyl estradiol causes an alteration in hydrocortisone metabolism.

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Received June 19, 1957. P.S.E.B.M., 1957, v95.

Determination of α -Amino Acid Nitrogen in Urine. (23370)

CHARLES SOBEL, RICHARD J. HENRY, NEIL CHIAMORI AND MILTON SEGALOVE
Bio-Science Laboratories, Los Angeles 64, California

The gasometric-ninhydrin method(1,2) is generally conceded to be the most specific technic available for determination of α -amino acid nitrogen in urine, but is rather tedious for routine analyses. The gasometric-nitrous acid(2,3) and the formol titration methods (2,4) are less specific. Folin's photometric technic(5) employing sodium β -naphtho-

quinone-4-sulfonate reacts with uric acid and ammonia present in urine(6,7). Cagan and coworkers(8) boiled off ammonia and used charcoal to remove uric acid, but did not investigate the specificity of their technic. Pope and Stevens(9) introduced a method wherein copper is bound to the amino group and the bound copper quantitated by iodometric titra-

tion, a technic applied to urine by Albanese and Irby(10). Photometric determination of the copper with sodium diethyldithiocarbamate has been introduced(11,12,13) but not applied to analysis of urine. It was believed worthwhile to investigate the specificity of the technic of Cagan, *et al.* and a modified photometric copper technic. Preliminary isolation of the amino acids on Dowex 50 x 8, a resin used by Stein(14) in chromatographic separation of urinary amino acids, enhanced the specificity.

Methods. *Isolation by ion exchange column.* Dowex 50 x 8, 200-400 mesh, analytical reagent grade, hydrogen form, is suspended in water and poured into a small column (10 mm internal diameter), in the bottom of which has been placed a glass wool plug. The column is packed by gravity to a depth of about 50 mm and then a glass wool plug placed on top of the resin. Ten ml of the urine is acidified to a pH of 1 to 2 (pH paper) with 1 N HCl and then passed through the column (the liquid level is never allowed to pass the upper surface of the upper glass wool plug). The column is washed with two 1 ml aliquots and one 3 ml aliquot of 0.01 N HCl, then with distilled water until the eluate is approximately pH 5 by pH paper. Add 1 ml of 1 N NaOH and, after the level has reached the top of the upper plug, add 10 ml of 1 N NaOH. When the NaOH front has descended to about the middle of the resin bed (detectable as a color change in the resin from a light brown in the hydrogen form to a darker brown in the sodium form), *i.e.*, after about 3 ml of eluate has appeared, begin collecting eluate in a graduated centrifuge tube to a total volume of 10 ml.

Copper method. *Reagents.* 1. Cupric chloride, 2.8% $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, filtered. 2. Sodium phosphate, 6.85% $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$, filtered; discard if turbidity develops. 3. Borax solution, 1.9% $\text{Na}_3\text{BO}_3 \cdot 10\text{H}_2\text{O}$, filtered. 4. Copper phosphate suspension. To 40 ml of the sodium phosphate solution add, slowly with constant shaking, 20 ml of the cupric chloride solution. Centrifuge and discard the supernate. Stir up the precipitate in about 15 ml of the borax solution, using a glass rod to break up the clumps. Add an ad-

ditional 45 ml of the borax solution, mix, centrifuge and discard the supernate. Repeat this washing procedure. Finally, suspend the precipitate in 100 ml of the borax solution containing 3 g NaCl. This suspension is usable for several weeks if kept in the refrigerator. This is the reagent as proposed by Schroeder(15) except for alteration in the sodium chloride content. The 5% concentration of NaCl used by Schroeder led to clumping of the suspension in a few days, rendering it unusable. 5. Sodium diethyldithiocarbamate, prepared freshly by dissolving 40 mg of reagent in 2 ml of distilled water and filtering. 6. Standard. Transfer 0.268 g glycine, 0.525 g glutamic acid and 1 g sodium benzoate to a 500 ml volumetric flask. Add about 200 ml water and shake to dissolve the solids. Add 4 ml conc. HCl, dilute to mark and mix. One ml contains 0.2 mg α -amino acid nitrogen.

Procedure. For greatest accuracy, or if the urine contains abnormally large amounts of ammonia, aerate the column eluate for one-half hour to remove the ammonia. Transfer 2.5 ml of column eluate to a 35 ml glass-stoppered centrifuge tube. Add 2.5 ml water, a drop of thymolphthalein indicator and add 6 N HCl drop by drop until indicator is decolorized, then add 1 N NaOH drop by drop until indicator just turns blue. A reagent blank is prepared by transferring 5 ml water to a similar tube, adding a drop of indicator followed by 0.1 N NaOH dropwise to the color change. A standard is prepared with 2.0 ml of the standard amino acid solution, 3.0 ml water, a drop of indicator and adding 1 N NaOH dropwise to the color change. To each tube, add 5.0 ml copper phosphate suspension, stopper and shake for 5 minutes. Centrifuge and filter the supernate through Whatman No. 1 filter paper. Dilute 1.0 ml of each filtrate to 25 ml with water. Transfer 2.0 ml of each diluted filtrate to a test tube. Add 0.1 ml of the diethyldithiocarbamate reagent, 3.0 ml isopropyl alcohol and mix. Read the absorbances of the unknowns (A_x) and standard (A_s) against the reagent blank at 435 $m\mu$. The color is stable for at least 2 hours and obeys Beers law, employing either a Beckman Model B spectrophotometer or a

Klett-Summerson photometer with a number 42 filter.

$$\text{Calculation: } \frac{A_x}{A_s} \times 16 = \text{mg } \alpha\text{-amino acid}$$

N per 100 ml urine.

β -Naphthoquinone - 4 - sulfonate method. The method of Cagan *et al.* was applied to the column eluate, including removal of ammonia by boiling but deleting the charcoal treatment since uric acid has already been removed by the ion exchange procedure.

Gasometric ninhydrin method. The technic of Van Slyke *et al.* (1,2) was employed except that, to effect a complete transfer of the CO_2 , it was found necessary to wash back and forth 12 times from the reaction tubes used to the Van Slyke chamber.

Results. Stoichiometry of the 3 methods. Taurine, glycine, histidine and 3-methyl histidine account for about 70% of the free amino acids normally excreted in the urine (14). Cystine, lysine, arginine and phenylalanine are also of interest because of increased excretion in certain metabolic diseases. Table I compares the apparent α -amino acid N yielded by these amino acids, employing the 3 methods applied directly to pure standard solutions. The relative values observed for the copper phosphate method do not agree exactly with similar studies by others (9,11). Examination of the relative values leads to the prediction of lack of exact correlation between the 3 methods.

TABLE I. Relative Values Obtained by the 3 Methods on Pure Standards.

	Relative values*		
	Gasometric ninhydrin	Copper phosphate	Naphtho- quinone
Glycine	.95	.93	.81
Histidine	1	1.28	.99
1-Methylhistidine	1	1.24	1.07
Phenylalanine	1	.95	1.10
Lysine	1.05	.89	1.33
Arginine	1	.91	.93
Taurine	.0	.0	1.06
Cystine	1.9	†	1.64

* Gasometric ninhydrin method—mols of CO_2 recovered per mol of amino acid. Copper phosphate and naphthoquinone methods—values are relative to a mixed glycine-glutamic acid reference standard, all in equimolar concentration.

† Low and variable.

Recovery of amino acids from ion exchange column. Recoveries of pure standards of glycine, alanine, glutamic acid, histidine, 1-methylhistidine, lysine, phenylalanine, arginine and cystine ranged from 94 to 104% as measured by the gasometric, copper phosphate and naphthoquinone methods. Recovery of amino acid nitrogen by column treatment of urines as determined by the gasometric technic ranged from 81 to 97% with a mean of 87% (10 urines, Table II). Recovery of taurine from the column, however, was less than 5%. This loss by column treatment would not be detectable by the gasometric technic in any case since taurine is not measured by this method.

Column vs. batch operation with resin. Removal of amino acids from urine by batch adsorption gave up to 50% less recovery than column treatment even when 2 successive batch treatments were employed.

Removal of uric acid and protein by column treatment. Uric acid gives color in the naphthoquinone procedure and is removed by charcoal adsorption in the method of Cagan *et al.* (8). They observed no loss of amino acid in the cases of glycine, glutamic acid and casein hydrolysate as long as the amount of charcoal (Norit A) employed did not exceed 100 mg per 100 ml of solution. Awapara and Sato (16) used charcoal (Darco G-60) adsorption as a purification step prior to chromatography of urinary amino acids. They added chloroform to prevent adsorption of tyrosine, phenylalanine and tryptophan and claimed that no loss of amino acids occurred. Following the technic of Cagan and coworkers, and employing 50 mg Norit A, losses of 20 to 60% were observed with arginine, cystine, glycine, glutamic acid, histidine, lysine, proline and serine added to urine. Losses amounting to 70 to 95% were observed with tyrosine and tryptophan. Use of Norit A washed with ether-acetic acid and then ether still gave large losses of tyrosine and tryptophan but gave considerably better recoveries of the other amino acids (losses of 0 to 20%). Using the technic of Awapara and Sato and Darco G-60, losses of 15 to 62% were observed with tyrosine, tryptophan, arginine,

TABLE II. Comparison of the 3 Methods, with and without Ion Exchange Treatment.

Nature of donor	mg α -amino acid N per 100 ml urine					
	Gasometric method		Copper method		Naphthoquinone method	
	Without resin	With resin	Without resin	With resin	Without resin	With resin
Normal adults	18.4	15.0	20.6	13.1	19.5	13.3
	8.2	6.8	12.6	7.0	13.7	6.5
	5.2	4.7	5.5	2.6	7.2	6.4
	16.2	13.2	25.0	14.7	27.1	19.5
	18.5	15.5	27.6	22.7	27.8	19.0
	10.5	9.9	12.8	8.8	14.7	9.9
	13.4	12.5	27.9	14.8	22.7	17.5
	10.3	10.0	16.3	10.6	16.8	12.8
	19.6	15.9	34.7	21.3	24.7	18.4
		7.3		7.0		8.7
		20.2		24.6		21.1
		12.2		18.6		12.9
		7.6		8.3		11.0
		10.6		10.2		13.0
		15.1		16.2		20.3
		9.7		14.4		12.3
		5.7		2.6		5.4
	14.4			13.6		14.7
	12.1			15.2		15.9
	18.7			27.0		18.1
	20.0			21.8		20.0
Child	4.0		2.0	2.8	6.5	2.9
"		8.3	19.0	10.2	11.8	6.7
Cirrhosis	2.9			1.8		5.1
"	6.3		21.5	13.5	18.7	14.5
Wilson's disease	15.0			14.9		13.4
" "	27.8			39.3		27.7
" "	17.5		20.2	18.8	24.0	17.1
" "	25.0		29.2	30.8	25.3	25.3
" "	25.3	23.1	44.5	36.0	35.0	27.0

glutamic acid, histidine, glycine, lysine, proline and serine, either as pure solutions or internal standards added to urine.

It was found that approximately 95% of uric acid was removed by the ion exchange column procedure when the initial uric acid concentration was as high as 85 mg %. From a study of the color produced by uric acid in the naphthoquinone reaction, it was concluded that a final concentration of 5 mg uric acid per 100 ml of column eluate would introduce an error of about 1% at a level of 20 mg α -amino acid N per 100 ml urine.

Experiments also showed that serum proteins added to urine to a concentration of 1% were effectively removed by the ion exchange column procedure.

Effect of heating. Only about one-third of the amino acids excreted are present in the urine in the free form, the remainder being conjugated(14). It is commonly presumed

that the 3 technics employed in this study determine the unconjugated α -amino acid N, preliminary acid hydrolysis being required if the total is desired. In the gasometric-ninhydrin method, the reaction is carried out at pH 2.5 and 100°C for 8 minutes(2) and, in the β -naphthoquinone method, the ammonia is removed from the urine by boiling at a pH of about 9.5 for 15 minutes. Several urine samples were analyzed, employing these conditions, and also doubling reaction time with ninhydrin and removing the ammonia by vacuum distillation at 50°C for the β -naphthoquinone method. No differences in results were observed, indicating no hydrolysis of the conjugated amino acids present.

Interference by ammonia in the copper phosphate method. Albanese and Irby(10) claimed that urea, uric acid, creatine, creatinine and ammonia do not interfere in the copper phosphate method. In view of the fact that ammonia complexes copper in

alkaline solution this seemed implausible. This was reinvestigated and it was found that, by the technic proposed, a urine concentration of 0.12% NH_3 gave a color in the copper phosphate method equivalent to 1.7 mg α -amino acid N per 100 ml. Since the normal output of ammonia is about 0.7 g per day(17), the positive error in a urine with normal ammonia content is about 1 mg % if the ammonia is not removed. If the ammonia is high as a result of disease or degradation of urea on standing, the ammonia should be removed. It was found that an ammonia concentration of 1% in the original urine was effectively removed by the aeration technic suggested.

Comparison of results obtained by the 3 methods. Table II lists the results obtained in a comparison of the 3 methods, with and without preliminary ion exchange treatment. Each value given in the table is the mean of duplicate determinations. In the case of the naphthoquinone method applied to urine not treated by ion exchange, uric acid was removed by charcoal adsorption by the procedure of Cagan *et al.* It is seen that results obtained by the copper phosphate and naphthoquinone methods without ion exchange treatment ranged up to double those obtained by the gasometric ninhydrin method. Thus, in spite of loss of amino acids in the charcoal treatment for removal of uric acid, the naphthoquinone method without the ion exchange isolation yielded falsely high results in several instances. Preliminary isolation of the amino acids by ion exchange significantly improved correlation of results given by the 3 methods.

Reproducibility. The standard deviations calculated from the duplicate determinations were 0.51, 0.68 and 0.81 mg % for the gasometric, copper phosphate and naphthoquinone methods, respectively.

Stability of urine samples. Four normal urine samples held at room temperature were analyzed for α -amino acid N daily for 4 days with no change indicated by any of the 3 methods. After 1 week standing, 3 of the samples appeared unchanged, whereas the fourth urine showed an increase of about 50%. By all 3 methods, the same 4 urines were

found to be stable for 5 weeks at refrigerator temperature.

Discussion. In the spectrophotometric technics of Woiod(12) and Beauchene and coworkers(13) the color complex produced with diethyldithiocarbamate, which is relatively water-insoluble, is extracted into amyl or isoamyl alcohol. In the present technic, this extraction is obviated by the addition of isopropyl alcohol to the aqueous solution.

By preliminary isolation of the urinary amino acids by ion exchange resin, taurine is lost because it is not adsorbed by the technic used. This is of concern only in the case of the β -naphthoquinone method since the other 2 methods do not determine taurine. This would tend to bring the 3 methods into better agreement. Normally, about 12 to 20% of the total free amino acid N is present as taurine(14,18). It is obvious, however, that the 3 methods under consideration will not give exactly the same values. Nevertheless, these results can be of value for clinical purposes.

From the results obtained in this study, the choice between the copper phosphate and naphthoquinone methods applied to urines after preliminary isolation of the amino acids by ion exchange appears to be one primarily of convenience. It is the authors' opinion that for the occasional sample the naphthoquinone method is somewhat more convenient but for extended series of samples the copper phosphate method is somewhat simpler.

Summary. A modification of the photometric copper phosphate method for determination of α -amino acid nitrogen in urine has been presented. A comparison of results obtained in urines by the copper phosphate, naphthoquinone and gasometric-ninhydrin methods indicates that the specificity of the copper phosphate and naphthoquinone methods is materially improved by preliminary isolation of the amino acids on an ion exchange column.

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Received June 21, 1957. P.S.E.B.M., 1957, v95.

Histology and Regenerative Capacity of Liver Following Multiple Partial Hepatectomies.* (23371)

DWIGHT J. INGLE AND BURTON L. BAKER

Ben May Laboratory for Cancer Research, University of Chicago, Chicago, Ill., and Department of Anatomy, University of Michigan Medical School, Ann Arbor

Repeated partial hepatectomies were performed in the rat in order to study in the remaining portion of the liver (a) maintenance of regenerative capacity, (b) histological alterations, and (c) possible appearance of neoplasia as a result of prolonged stimulus to regeneration. The regenerative capacity was not exhausted by as many as 12 partial hepatectomies and neoplasia was not seen.

Procedure. The surgery was carried out at the University of Chicago and the histological studies were done at the University of Michigan. The procedure for repeated partial hepatectomy in the rat has been described(1). Thirty male rats having initial weights of 240-260 g were given Archer Dog Pellets to eat *ad libitum* during the experiments. Twelve of the rats were subjected to sham operations which consisted of a laparotomy once each month. Eighteen rats underwent repeated partial hepatectomies, once each month. The procedure was to remove all of the liver but

the median lobe at the first operation, a part of the median lobe was excised at the second operation and thereafter liver was aspirated by negative pressure applied to a pipette inserted in a slit in the now thickened capsule of liver. The post-operative care of the rats has been described(1).

At the termination of the experiment, the rats were killed by exsanguination under ether anesthesia. The liver was fixed in ice-cold Carnoy's fluid and left in the refrigerator overnight. On the next day the Carnoy's fluid was replaced with absolute alcohol and the livers shipped to Ann Arbor. A large piece of the liver was prepared for microscopic study. Sections were stained with periodic acid-leucofuchsin(PAS) and counter-stained with methylene blue in a citric acid-phosphate buffer (pH 5.6).

Results. All of the sham-operated rats survived for the duration of the experiment. All of the hepatectomized rats survived 8 operations. During the following 4 months 5 rats died as the result of self-inflicted wounds after the incision of the last operation had healed. Two rats died from post-operative hemorrhage due to breaking of adhesions. There were no deaths due to failure to control hemor-

*Supported in part by research grants from the N.I.H., Public Health Service, the University of Michigan-Memorial Phoenix Project No. 57, and The Upjohn Co. to Dr. Baker, and by a grant from the American Cancer Society as recommended by the Committee on Growth.

TABLE I. Body Weights and Liver Weights of Sham Operated Rats and Rats Subjected to 12 Partial Hepatectomies during 1 Year. Averages and stand. errors.

Group	No. rats	g body wt		g liver wt	
		Initial	Final	Total removed	Final
Sham operated	12	250	534 $\pm$ 15.3		17.5 $\pm$ 1.15
Partial hepatectomy	6	250	507 $\pm$ 20.8	70.77 $\pm$ 1.54	13.8 $\pm$ 1.22

rhage from the liver itself. The remaining complications represented abscesses and diffuse infections which were no longer controlled by penicillin and streptomycin. After 8 months all of the rats developed peritonitis on one or more occasions following liver surgery. All of the rats which were killed because of impending death had multiple abscesses in the remaining liver and elsewhere in the peritoneal cavity and occasionally in the lungs. Gastric ulcers were found in 3 of these rats.

As previously noted(1) the rat subjected to repeated partial hepatectomy shows various signs of hepatic insufficiency during the first week following each operation. In addition to lowered resistance to infection these rats develop hypoglycemic shock unless given readily utilizable carbohydrate. Blood clotting is impaired and the tiny broken or cut vessels of adhesions or scars may continue to leak slowly for hours. The rats usually became jaundiced for a few days following operation.

Six hepatectomized rats survived for the duration of the experiment (1 year) and in them the liver was reduced 12 times. Data on these rats and the sham-operated controls are in Table I. The body weight of these animals was maintained rather well. One rat did lose 121 g during the 12th month after gaining weight at a normal rate during most of the year. At autopsy it was found to have severe bronchiectasis. The capacity of liver to regenerate was also maintained in spite of the removal of large amounts of liver during the year although the final average weight was somewhat less than normal.

The histology of the regenerated liver from the 6 rats which lived throughout the experiment was similar with respect to several points (Fig. 1 and 2). No mitosis was observed in parenchymal cells. This was to be expected because the most active proliferation

occurs within a few days after the operation (2). A normal relationship between parenchymal cells and sinusoids was maintained. However, the pattern of orientation of liver cords within lobules was not as regular as in livers from sham-operated rats. In all cases there were areas of parenchymal cell necrosis and of fibrous scars which usually contained proliferated bile ducts. In one case, septa of connective tissue fibers divided the liver substance into compartments. Abscesses were not found in the livers of these rats, suggesting again that the capacity to resist infection was an important factor in determining duration of survival after repeated partial hepatectomy.

Cytologically there was considerable variation between the individual animals. Where-

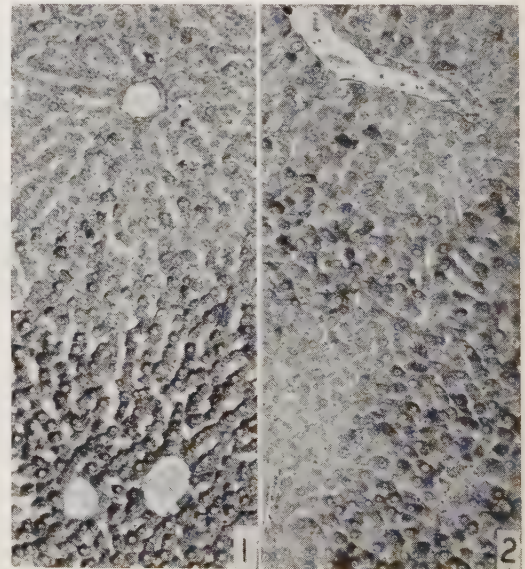


FIG. 1. Liver from sham-operated control. Cytoplasmic material appearing black is glycogen; most of the gray bodies are ribonucleic acid. Portal canal is at the top and central vein below. PAS and methylene blue. $\times 125$.

FIG. 2. Liver from rat which was partially hepatectomized 12 times. PAS and methylene blue. $\times 125$.

as in the specimens from sham-operated rats, cytoplasmic ribonucleic acid (as shown by staining with methylene blue) was generally in the form of dense clumps, in 3 of the hepatectomized rats it was diffusely distributed throughout the cytoplasm as a delicate stippling. This pattern seemed to be correlated with the presence of enlarged parenchymal cells and variability in nuclear structure. Some nuclei were enlarged and often contained either multiple nucleoli or hypertrophied nucleoli of variable shape. In one case, parenchymal cells were extensively vacuolated, indicating that fat had accumulated. The glycogen content did not follow a consistent pattern, in at least 3 cases being comparable in amount to that in the sham-operated controls.

Histologic study of the livers of the rats which died or were killed before termination of the experiment confirmed the frequency of infection in these rats by the presence of abscesses in the fibrotic scarred areas and by

mononuclear cellular infiltration into the connective tissue of the portal canals of some rats. Otherwise the histologic changes varied only in degree from those described for the rats which lived throughout the experiment.

In no case was histologic evidence of neoplasia observed.[†]

Summary. The capacity of liver to regenerate was maintained in rats which were subjected to partial hepatectomy 12 times within a period of one year. At the end of the experiment, only minor cytological changes were observed in the regenerated liver and there was no neoplasia.

[†] We are grateful to Dr. M. R. Abell, Dept. of Pathology, Univ. of Michigan, for his analysis of 2 specimens.

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Received June 25, 1957. P.S.E.B.M., 1957, v95.

Intraperitoneal Insemination of the Heifer.* (23372)

LESLIE E. McDONALD,[†] AND JESSE SAMPSON

*Department of Veterinary Physiology and Pharmacology and Agricultural Experiment Station,
University of Illinois, Urbana*

Anatomically, there is no barrier to prevent sperm from reaching the ovary following intraperitoneal injection of semen in species such as the cow, ewe, mare(1), rabbit(2), and to some extent the sow(1) whereas bursae such as those of the rat(2), bitch(1), and mouse(3) could be a barrier. Physiologically, little is known of the compatibility of the peritoneal surfaces and fluid with sperm. Brown(4) found peritoneal fluid of the human being compatible with living sperm. Van Drimmelen(5) obtained fertilization in the chicken following intraperitoneal insemina-

tion. Black *et al.*(6) failed to obtain fertilization when 9 superovulated calves were inseminated intraperitoneally. Skjerven(7) recently reported conception in a heifer following intraperitoneal deposition of semen by puncturing the vaginal wall.

It is the object of this paper to report the results of intraperitoneal insemination of heifers.

Materials and methods. Four sexually mature Holstein-Friesian heifers were inseminated a total of 6 times near the end of estrus by injection of Holstein-Friesian semen, in some cases diluted, in other cases undiluted and of variable quality, into the peritoneal cavity by puncturing the abdominal wall in the region of the right paralumbar fossa with a 16-gage, 4 inch hypodermic needle. An in-

* We are indebted to the Department of Dairy Science for supplying the semen used in this study.

[†] Present address: Oklahoma State University, Stillwater. Published with the approval of Director of the Ill. Agri. Exp. Station.

TABLE I. Pregnancy in the Heifer following Intraperitoneal Insemination.

Heifer	Day of insemination	Semen	AP extract (ml)	Pregnancy
146	4/24/53	Undiluted, 2 ml, 12 hr old	none	no
145	4/28/53	Diluted, 2½ ml, 1 day old	"	"
826	5/ 8/53	Undiluted, 1½ ml, 12 hr old	2	yes
148	5/10/53	Undiluted, 5½ ml, 2 days old	2	no
146a	5/12/53	Diluted, 1 ml, 1 day old	2	"
145a	6/ 8/53	Diluted, 2 ml, age unknown	none	"

travenous injection of anterior pituitary extract was given to 3 heifers at the time of insemination. Periodic rectal palpations of the uterus were made following insemination to determine if conception occurred. Bulls were not kept on the experimental farm or on adjacent farms during the experiment. The heifers were exercised in a small pasture and observed daily.

Results. One of 6 intraperitoneal inseminations was followed by pregnancy. The gestation length of 275 days is within one standard deviation of the mean gestation length for the breed(8).

Discussion. Experimental insemination of the mammal by the intraperitoneal route offers a method of studying the processes leading to fertilization. Chang(9) found an aging process of sperm while transgressing the female tract necessary to induce "fertilizing capacity" in rabbit sperm. In contrast to this, Runner(2) injected sperm into the ovarian bursae of mice, by-passing sperm migration through the uterus, and obtained con-

ception. Data reported herein indicate that sperm may not need to transgress the tubular genital tract of the cow in order to be capable of fertilization; although it is possible, in our experiment, that fertilization occurred in the tubular genital tract after the fertilizing capacity of the sperm had developed. The peritoneal surface and fluid permitted sperm viability in at least one animal, indicating compatibility.

Injectations of anterior pituitary extract were made in order to hasten ovulation as demonstrated by Marion *et al.*(10). A shortened insemination ovulation interval should encourage fertilization if the sperm were weakened by an abnormal environment.

Summary. Intraperitoneal insemination of 4 heifers a total of 6 times resulted in one pregnancy. This experimental method is offered as a means of studying the physiological processes leading to fertilization.

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Received July 1, 1957. P.S.E.B.M., 1957, v95.

Lipogenesis by Adipose Tissue, Dietary Effects. (23373)

J. P. MILLER,* JOHN A. D. COOPER, AND SMITH FREEMAN

Department of Biochemistry, Northwestern University Medical School, Chicago, Ill.

The effects of dietary factors upon lipogenic activity of adipose tissue have been reported by several investigators(1-4). Tuerkischer and Wertheimer(1) reported that fat deposition in adipose tissue of fasted rats is apparent one day after the beginning of recovery feeding. Good correlation was found between glycogen deposition and fat accumulation in adipose tissue. Shapiro and Wertheimer(2) demonstrated that adipose tissue incubated *in vitro* in serum enriched with deuterium oxide was capable of introducing stably-bound deuterium into its fatty acids. The rate of introduction was greater in the adipose tissue of rats on a diet accelerating fat synthesis in the body. In investigations of dietary effects on utilization of radioglucose by adipose tissue *in vitro* Hausberger and Milstein(3) found that fasting or feeding a high fat diet abolished lipogenesis and greatly diminished glucose oxidation. The "block" in fatty acid synthesis by adipose tissue from fasted animals was studied by Rose and Shapiro(4) and led them to the conclusion that the pathway of glucose metabolism is changed during fasting to one which does not facilitate fat synthesis. The present report supplements previous knowledge of dietary effects upon lipogenic activity of adipose tissue and, in addition, provides information concerning lipid synthesis as a function of time after recovery feeding and tissue composition.

Procedures. Minced rat (Sprague-Dawley) epididymal adipose tissue plus 3 ml normal dog serum and 100 μ l labelled acetate solution (2.38 μ m acetate) was incubated for 3 hours at 37.5°C in a 10 ml flask containing a center well tightly stoppered with a rubber serum stopper. Following incubation, 0.4 ml Hyamine(5) solution was injected into the center well, followed by 0.5 ml 6N acetic acid into the main compartment of the flask. After equilibration at 37.5°C for 1½ hours the

Hyamine solution was quantitatively collected for counting. The contents of the flask were then saponified, made acidic, extracted with petroleum ether and the lipid extract chromatographed by the procedure described by Popjak and Tietz(6) to rid it of contaminating traces of C¹⁴-acetate. All samples were counted in a liquid scintillation counter.† Carbon dioxide samples were counted in a system consisting of Hyamine solution (combined with CO₂) and 0.3% diphenyl-oxazole (DPO)‡ in toluene(5). All other samples were counted in a system consisting of 2% water, 25% absolute ethanol, 73% toluene (containing 3 g per liter DPO).

Analysis of the nitrogen content of adipose tissue was carried out by micro-Kjeldahl steam distillation into a boric acid-mixed indicator solution and subsequent titration with standard HCl. A mixture of H₂SO₄, CuSO₄, K₂SO₄ and Se was used for digestion. Lipid content was estimated gravimetrically on a petroleum ether extract of tissue digested with 2N H₂SO₄. Reducing substances were determined by a modification of the Folin method (7) on a lipid-free digest of the adipose tissue. Quantitative recovery of glucose added to adipose tissue was obtained with the method. The results are expressed in terms of glucose equivalent.

Results. A group of 9 normal rats on Rockland rat diet were divided into 2 groups. Three animals were sacrificed and served as the source of adipose tissue for control values. The remainder were fasted with water *ad libitum* to a loss of approximately 20% of their initial weights, which required about 48 hours. The animals were then refed on a high carbohydrate diet§ *ad libitum*. Rats were sacrificed at intervals and adipose tissues obtained. The tissue was analyzed for nitrogen, glucose, lipid content and rate of conversion of 1-C¹⁴ ace-

* Present address: Nutrition Department, Abbott Laboratories, North Chicago, Ill.

† Tri-Carb Counter, Model 314, Packard Instrument Co., LaGrange, Ill.

‡ Pilot Chemicals, Inc., Waltham, Mass.

TABLE I. Lipid Synthesis as a Function of Time after Refeeding and Tissue Composition. Normal rats

Animal	Condition	% wt loss (before refeeding)	Tissue composition			% of total acetate incorporated per 100 mg tissue	
			% nitro- gen	% lipid	% glu- cose	CO ₂	Lipid
1	Normal (before fasting)	—	.27	87.3	.10	1.46	9.39
2			.30	88.0	.32	—	—
3			.29	82.4	.08	2.10	12.38
		Avg	.29	85.9	.17	1.78	10.89
4	0 hr after refeeding	20.4	.45	69.3	.14	1.59	1.26
5	6 " "	20.3	.36	81.4	.09	1.98	1.00
						1.35 (avg 1.67)	1.01 (avg 1.00)
6	12 " "	21.4	.38	77.2	.13	1.97	5.69
							5.78 (avg 5.74)
7	18 " "	19.0	.42	76.6	.19	2.96	8.76
						2.32 (avg 2.64)	8.01 (avg 8.38)
8	24 " "	20.6	.51	68.1	.44	2.61	12.01
						3.36 (avg 2.99)	11.45 (avg 11.73)
9	48 " "	23.1	.89	59.1	1.03	3.48	17.70
						2.91 (avg 3.20)	15.79 (avg 16.75)

tate into CO₂ and long chain fatty acids *in vitro*. Analyses were usually done in duplicate or triplicate. The data obtained are given in Table I. In most cases duplicate studies were carried on in the *in vitro* metabolism of radiocarbon-labelled acetate for each animal studied. To show the reproducibility, these individual results are presented in the table with average values.

Fasting increases the nitrogen content of adipose tissue, probably in part by decreasing the lipid content. Upon refeeding the nitrogen content of the tissue increases even further. The reducing substances show no essential change on fasting or during the first 18 hours of refeeding but at 24 hours a sharp increase is apparent which continues during the next 24 hours. The results are in good agreement with those of Tuerkischer and Wertheimer(1) on fasted and refed rats. The depression of fatty acid synthesis *in vitro* from acetate following fasting confirms previous reports(3,4). Reduced lipogenic activity persists for 6 hours after refeeding but then fatty acid synthesis rises rapidly to the high level of incorporation of 16.8% of the labelled acetate added. Under the conditions of these experiments a considerably greater

conversion of acetate to fatty acid was obtained than Hausberger *et al.*(9) report with glucose. Fasting and refeeding exert a much smaller effect on the rate of conversion of acetate to C¹⁴O₂. It is interesting to note that increased fatty acid synthesis begins before an appreciable rise in tissue reducing substances occurs, apparently glycogen content does not rise first as Tuerkischer and Wertheimer(1) inferred. The lipid content of the tissue declines during the refeeding period in spite of an increased rate of synthesis of fatty acids. The mobilization of fat evidently is proceeding at a rate which exceeds that of synthesis. Because of the high fat content of adipose tissue it was not possible to determine the extent to which net fat synthesis occurred.

Feller(10) studied incorporation of C¹⁴-l-acetate into fatty acids with normal mouse adipose tissue in an *in vitro* system consisting of Krebs-bicarbonate buffer supplemented with succinate and glucose. On a fat-free basis he found an even greater per cent acetate incorporation per 100 mg tissue in adipose tissue than in liver (2.51% as compared to 1.24%). Under more favorable circumstances (fasted and refed rat adipose tissue incubated in serum) the present study has demonstrated levels of incorporation of acetate into lipid as great as 40% per 100 mg of fat-free adipose tissue (Table I).

§ Osborne-Mendel salt mixtures(8) 3.83%; wheat germ oil 0.29%; cod liver oil 0.29%; cellulose 9.55%; corn oil 0.77%; sucrose 67%; casein 14.35%; dried brewer's yeast 3.91%.

During this work the lipogenic activity of adipose tissue from B-complex deficient rats has also been studied. The per cent of total acetate incorporated into lipid per 100 mg tissue in the deficient rats averaged 1.59% (CO₂ formation 1.53%). One week following supplementation of the diet with B-complex vitamins, lipogenic activity of adipose tissue from these rats was found to average 19.08% (CO₂ formation 0.81%). Tuerkischer and Wertheimer(1) have found that addition of dried yeast to the diet of B-complex deficient rats will cause deposition of adipose tissue glycogen.

Summary. 1. The incorporation of C¹⁴-acetate into lipid and carbon dioxide by rat adipose tissue (*in vitro*) was investigated. This investigation concerned the effect of the nutritional status upon the lipogenic activity of adipose tissue. 2. Fasting was found to produce an increase in nitrogen concentration and a decrease in lipid concentration and lipogenic activity. 3. In normal rats lipogenic activity of the tissue was found to increase during recovery feeding following fast-

ing. The increased activity was paralleled by increased nitrogen and glucose content of the tissue and by decreased lipid content. 4. Lipogenic activity of adipose tissue of rats could be made relatively constant by depletion of fat stores followed by recovery feeding.

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Received July 2, 1957. P.S.E.B.M., 1957, v95.

Effect of Pituitary Growth Hormone on Transplantable Mouse Tumors. (23374)

E. A. MIRAND AND J. G. HOFFMAN

Roswell Park Memorial Institute, Buffalo, N. Y.

In a recent study of the effect of growth hormone on a mammary adenocarcinoma transplantable in DBA/1 mice, Mirand(1) and Mirand and Hoffman(2) reported that tumor volume was significantly increased when the host mouse was given hormone beginning at the time of inoculation. Reid's (3) review indicates that mouse tumor response to growth hormone was not a consistent finding. We examined this problem further with more quantitative experiments on 4 mouse tumors using purified growth hormone which we assayed for its effect on hypopituitary dwarf mice(4,5).

Materials and methods. The effect of growth hormone was studied on 4 transplant-

able tumors. 1. The DBA/1 mammary adenocarcinoma which originated spontaneously in the DBA/1 mouse, 2. the Marsh Albino (MA) mammary adenocarcinoma which originated spontaneously in the MA mouse. 3. and 4.: Tumors 3 and 4 were fibrosarcomas induced in the DBA/1 and MA mouse strains respectively by desoxycorticosterone acetate in sesame oil(6). These 4 tumors grew in castrated mice indicating their lack of hormonal dependence on the host. All tumors were grown subcutaneously in male mice having an average age of 3 months at the time of inoculation. Cell suspensions of the tumors were made by the method of Reinhard, Goltz and Warner(7).

TABLE I. Assay of a Purified Beef Growth Hormone Preparation (Horner's)* in Adult Hypopituitary Dwarf Mice† by Body Weight.

Dwarf mouse No.	Wt in g				
	Initial	After 1 wk .4 mg/day	After 2nd wk .6 mg/day	After 3rd wk 1 mg/day	After 4th wk 1.5 mg/day
1—♂	11.3	15.1	20.5	22.5	25.5
2—♂	8.7	14.5	19.5	23.5	26.5
3—♂	11.0	17.5	21.5	25.5	28.5
4—♀	8.5	13.5	17.5	20.5	23.5
Avg	9.9	15.2	19.8	23.0	26.0
Controls, saline inj.					
1—♂	11.0	10.5	11.5	12.0	12.0
2—♂	10.5	11.0	12.5	11.0	12.0
3—♀	8.5	9.0	9.0	9.5	9.0
Avg	10.0	10.2	11.0	11.0	11.0

* Growth hormone preparations contaminated with ACTH showed no growth promoting action after the 4th wk in hypopituitary dwarf mice. Growth hormone preparation contaminated with TSH after the 4th wk gave an avg body wt value of 38 ± 2.5 g in hypopituitary dwarf mice.

† Syracuse Strain.

A purified beef growth hormone preparation (Lot #GE-5-F. W. Horner Ltd., Montreal, Canada) was given immediately after tumor cell inoculation and in increasing doses weekly according to the following schedule. In the first week at the time of tumor inoculation 0.4 mg per day, second week 0.6 mg per day, and third week 1.0 mg per day. On the eighteenth day the animals were weighed and sacrificed. The excised tumors from these animals were weighed by the water displacement method. Potency of hormone was assayed beforehand on hypopituitary dwarf mice that had attained their maximum adult body weight. Other growth hormone preparations (from Armour Co. and Princeton Laboratories) were assayed on hypopituitary dwarf mice, but were not used because a maximum growth response on dwarf mice was not evident. Growth hormone preparations contaminated with ACTH did not show growth promoting action on the hypopituitary dwarf. Growth hormone preparations contaminated with TSH gave a greater effect than the purified growth hormone preparation (Horner's) used in this study. Table I shows the increase of body weight in the hypopituitary dwarfs at 4 successive week intervals with progressively increased hormone dosage. Control dwarf mice were injected with saline, the same volume as given above. Results given in Table I show when growth hormone is given at least a 2-fold increase in body weight

of the hypopituitary dwarf mouse occurs in 4 weeks.

Results. Tables II A and B show the results for these tumors and the host mouse body weights when the animals were sacrificed on the eighteenth day after inoculation. They also show that variation of number of cells per inoculum by a factor of 50 had no significant effect on tumor growth, either in the final weight of tumor or in the observed latent period. The reasons for this lack of variation with inoculum size are unknown, but one important factor is probably the constancy of the final tumor mass attained in a given host mouse, as will be described below.

Comparison between the hormone treated animals and the controls, which were saline injected, shows that the host animal underwent approximately a 20% increase in body weight and that there is about a factor of 3 increase in tumor weight in the presence of administered hormone. Also, the tumor latent period is reduced by about 25%, a fact which points to an increased multiplication rate. In order to estimate the shortening of generation time, measurements were made of the tumor volume when it first became palpable. These showed it to be of the order of 2 cmm. This figure permits an estimate of the generation time(8,9) in the DBA/1 fibrosarcoma (Table II A) to be 14 hours in the controls. In the hormone treated animals the tumor cell generation time was 10 hours.

TABLE II A. Effect of Growth Hormone on Average Tumor and Host Weights. DBA/1 fibrosarcoma inoculated subcut. in DBA/1 males.*

Cells per inoculum	No. of animals	Hormone treated				No. of animals	Controls (saline inj.)			
		Body wt (g)		Tumor wt (g)	Latent period (days)		Body wt (g)		Tumor wt (g)	Latent period (days)
		Initial	Final				Initial	Final		
596	12	16.5	25.4	.25	11.7	12	17.2	21.3	.06	15.7
2112	12	16.6	25.2	.22	12.2	12	16.5	20.4	.07	15.7
5026	12	16.9	24.8	.24	10.8	12	16.7	19.8	.07	14.4
10200	12	15.5	23.8	.25	11.4	12	16.2	19.9	.07	15.3
25700	12	16.3	24.8	.23	11.1	12	15.3	19.3	.07	14.3

TABLE II B. DBA/1 mammary adenocarcinoma inoculated subcut. in DBA/1 males.*

		Hormone treated					Controls (saline inj.)			
		Initial	Final	Tumor wt (g)	Latent period (days)		Initial	Final	Tumor wt (g)	Latent period (days)
515	12	16.8	26.9	.48	12.9	12	17	22.2	.16	15.5
2010	12	16.7	27.0	.53	10.9	12	17.1	23.2	.28	13.8
4825	12	16.3	25.6	.49	11.4	12	16.5	22.4	.19	14.6
10850	12	16.7	25.5	.64	11.2	12	16.9	21.5	.18	15
26850	12	16.5	25.1	.73	10.5	12	17.3	21.4	.22	13.5

* All tumors and final mouse weights were measured on 18th day after inoculation.

However, the growth period from inoculation to sacrifice was not always fixed at 18 days. Experience showed that it was desirable to terminate growth of tumor at various times beginning as early as 11 days after inoculation depending on growth rate. For example, in the DBA/1 mammary adenocarcinoma, (Table II B), some of the tumors at 18 days had attained a volume of about 1 ccm. It was felt that such large tumors were undesirable for quantitative measurements because of possible limitation of vascular supply.

A variable growth time for the tumors made it necessary to introduce the host body weight as a basis of reference for comparison of the various experimental conditions. Since the mice had a spread of body weight, it was necessary to express the increase in body weight at any time after tumor inoculation as the ratio of final to initial body weight. Thus, in the various strains used, the ratio of final to initial body weight ranged from 1.00 to 1.80 in the hormone treated mice and from 1.00 to 1.5 in the saline treated controls. In the MA fibrosarcoma, which is the first entry in Table III, the average mass of the tumor in control mice was 0.08 g, while the body weight ratio varied from 1.1 to 1.4. These control tumors had growth times ranging from 14 to 22 days. The same tumor in hormone treated males averaged 0.15 g over a range of body weight ratio from 1.3 to 1.6.

The growth times were less than those in the controls and varied from 13 to 20 days. The hormone shifted both the tumor and body weights to values greater than those of the controls.

The other 3 tumor entries in Table III show the same pattern as that described for the MA fibrosarcoma. The last 2 entries are for the one tumor, the DBA/1 mammary adenocarcinoma, the experiments being carried out at different times of the year, namely May and August. The tumor masses are greater in the August run than in the May run both in the controls and in the hormone treated animals. While the tumor masses are greater, the approximate constancy with body weight ratio is maintained, and hormone influence is manifest in increased mass of both tumor and host.

The data for the fourth tumor, the DBA/1 fibrosarcoma, are presented graphically in Fig. 1 to illustrate the pattern of response seen in each of the entries of Table III. The abscissae are ratio of final to host body weight and are common to both Fig. 1 a and 1 b.

Discussion. The effectiveness of a purified beef growth hormone preparation is demonstrated in the assay on hypopituitary dwarf mice. Table I shows that average body weight of these mice is slightly more than doubled after 3 to 4 weeks of treatment. Each mouse receiving growth hormone underwent a

TABLE III. Average Tumor Weight as a Function of Ratio of Final to Initial Host Body Weight.

Ratio of final to initial body wt	1.0/ 1.099	1.1/ 1.199	1.2/ 1.299	1.3/ 1.399	1.4/ 1.499	1.5/ 1.599	1.6/ 1.69	1.7/ 1.8
<i>MA fibrosarcoma in MA mice</i>								
Tumor with GH (g)				.12	.17	.17		
No. of animals				4	13	7		
Control tumors (g)		.07	.08	.08				
No. of animals		4	19	1				
<i>MA mammary adenocarcinoma in MA mice</i>								
Tumor with GH (g)		.22	.21	.17	.19	.20	.23	
No. of animals		1	6	16	21	25	3	
Control tumors (g)		.07	.06	.07	.07			
No. of animals		19	40	12	1			
<i>DBA/1 mammary adenocarcinoma in DBA/1 mice (May run)</i>								
Tumor with GH (g)		.22	.24	.22	.22	.25	.26	
No. of animals		9	12	15	17	3	4	
Control tumors (g)		.09	.07	.09	.15			
No. of animals		6	23	28	1			
<i>DBA/1 mammary adenocarcinoma in DBA/1 mice (Aug. run)</i>								
Tumor with GH (g)		.45	.45	.59	.49	.56	.56	.69
No. of animals		1	1	6	11	19	8	13
Control tumors (g)		.25	.19	.18	.22	.23	.20	
No. of animals		1	7	18	22	7	2	

consistent increase in body weight during the 4 weeks' time during which the exogenous hormone supplemented the deficient pituitary hormone.

In the non-deficient mice the hormone effect was not so pronounced although clearly evident. The 60 males of the DBA/1 strain in Table II A, for example, saline injected had an average body weight increase of from 16.4 to 20.1 g in 18 days. The 60 males treated with GH increased from 16.3 g to 24.8 g in the same time interval.

More pronounced, however, is the effect of GH on the transplanted fibrosarcoma (Table II A) whose mass was increased by at least a factor of 3 over the saline injected controls. The results of Tables II A and B parallel the results on tumor fragment transplantation of Smith *et al.*(10) who found that GH increased both the body weight and tumor growth rate of C3H mice bearing a transplantable mammary adenocarcinoma. Evidence for increased growth rate in the DBA/1 fibrosarcoma is exemplified in the reduction of the average latent period from 15.7 days in controls to 11.7 days in the first run of 12 mice in Table II A.

No one of the 4 different tumors failed to

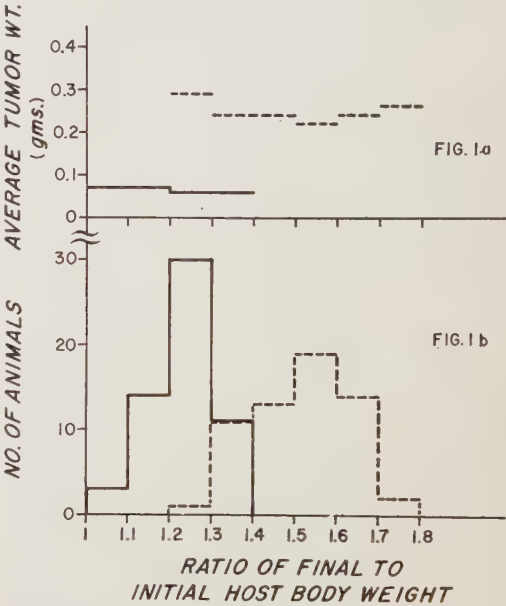


FIG. 1a. Avg tumor weights (ordinates) for corresponding ratio of final to initial host body wt (abscissal). DBA/1 fibrosarcoma in DBA/1 male mice, solid lines tumor wt in controls, saline inj. Dashed lines, tumor wt in mice treated with growth hormone.

FIG. 1b. Histogram of distribution of numbers of animals treated with growth hormone and controls, saline inj., plotted against ratio of initial to final wt of host body wt of tumor bearing animals in Fig. 1a.

show a response in increased growth. Referring, for example, to the fibrosarcoma in the DBA/1 males (Table II A) the average control tumor weighed 0.07 g compared to the average of 0.238 g in the GH treated mice. The mammary adenocarcinoma in DBA/1 males (Table II B) had a control weight averaging 0.21 g as compared with 0.57 g in the GH treated mice. The ratio of GH treated fibrosarcoma weight to its control is 3.4 whereas in the mammary adenocarcinoma the ratio is 2.7. In the MA strain its mammary adenocarcinoma had a higher ratio than did the fibrosarcoma (Table III). No significance is attached to the differences in ratios. Thus, the 4 tumor types studied all have about the same sensitivity to GH in either the DBA/1 or MA male mice.

The constancy of maximum attainable tumor mass in a given host is of special interest. In Tables II A and B the inoculum size varied from about 500 to 26,000 or by a factor of 50, yet the final tumors had the same average mass. This is especially notable in the fibrosarcoma where the 5 separate runs had average tumor masses from 0.22 to 0.25 g and the controls averaged 0.06 to 0.07 g. This is interpreted as meaning that regardless of inoculum size the tumors attained a fixed, characteristic mass and grew no further. By the eighteenth day all tumors had attained this average volume.

As the data of Table II and Fig. 1 show, the host body weight may increase from 10 to 60%, yet the average tumor mass remains at the level of the fixed characteristic value both in control and in GH treated mice. The graph in Fig. 1a summarizes the GH effect in displacement of the line for the average tumor mass. It is displaced upward because tumor size is increased, and to the right because average body weight of the host is also increased by GH. While host weight may increase 10 to 15% tumor average increases from a minimum of 50 to 300% depending on the tumor type and the host.

The complexity of growth hormonal action is indicated by many reports(1). There has been controversy as to whether the anterior pituitary hormones which promote or assist

to promote normal (and perhaps cancerous) mammary growth are due to specific mammo-genic hormones working in concert with ovarian hormones, or whether the hypophyseal contribution can be wholly attributed to the action of one or more of the 6 generally accepted and characterized anterior-pituitary hormones. The data in this report indicate that mammary tumor cells as well as fibrosarcoma cells can respond to growth hormone and that the effect is not entirely a general metabolic one. Pearson, *et al.*(11,12) reported recently the anabolic and symptomatic effects of human growth hormone in hypophysectomized patients with osseous mammary metastases. Their data suggested that pituitary growth hormone is an important factor for mammary carcinoma growth. From the reports of other investigators, and ours, it would seem reasonable to assume that growth hormone in some way plays a role in carcinogenesis(13-17) and in supporting neoplastic growth.

Summary. 1. Growth hormone was assayed for potency by its effect on body weights of adult hypopituitary dwarf mice. Their body weights increased by more than a factor of 2 during 4 weeks' administration of the hormone. 2. Four different transplantable tumors responded to growth hormone administered to the host mice. The latent period of the tumor is reduced and the mass of the tumor is much greater than that of the controls. 3. Tumor bearing host mice showed a significant body weight increase under action of growth hormone. Enhanced tumor growth due to the hormone did not reduce the host body weight. 4. Maximum average tumor mass attained by the 4 different tumors is independent of the host body weight. While growth hormone increases the tumor as well as the hosts weight, average mass of tumor grown is constant and characteristic of the tumor-host combination. 5. Data suggest that growth hormone plays a supportive role in mammary carcinoma.

The authors express their thanks to Dr. John Kent, Louise Schwabe, A. G. Mirand, Dr. Donald PoChedley and Alan Case for their technical assistance. This investigation was supported in part by research grants

from National Cancer Institute, U.S.P.H.S.

Thanks are extended to Dr. Irby Bunding of Armour Co. and Dr. Kleinberg of Princeton Laboratories for their generous donation of growth hormone preparations.

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Received July 2, 1957. P.S.E.B.M., 1957, v95.

Transplantable Mastocytoma in the Mouse Containing Histamine, Heparin, 5-Hydroxytryptamine.* (23375)

JACOB FURTH, PAUL HAGEN, AND EUGENIA I. HIRSCH

Children's Cancer Research Foundation, Children's Medical Center, and Departments of Pharmacology and Pathology, Harvard Medical School, Boston, Mass.

Mast cells are among the current problem cells of the body. Several vital functions have been attributed to them but the relation of any functions to the mast cells has not been firmly established. They are said to be the major, if not the sole, source of heparin (1); so they may be involved in the clotting mechanism. Their relation to connective tissue and their high content of heparin suggest that they may be involved in the elaboration of the mucopolysaccharides of the collagenous ground substance. They are a rich source of histamine(2) and may play a part in processes of inflammation and hypersensitivity. It has been reported that they contain 5-hydroxytryptamine (serotonin, 5-HT)(3), a naturally occurring amine with pharmacological actions on smooth muscle. The distribution of mast cells in certain locations and in

certain tumors apparently follows a definite pattern(4), the significance of which is not clear.

Tumors composed of mast cells are well known in animals; none has been reported in man. The most studied is that occurring occasionally in dogs. It was first described by Bloom(5) and its high heparin content established by Oliver *et al.*(6). Deringer and Dunn(7) have described a mouse mastocytoma. Transplantation of a mastocytoma, apparently carried out simultaneously with that described here, has just been reported by Dunn and Potter(8). A survey of the relevant literature is beyond the scope of this report.

Origin. The tumor was found in a 500-day-old mouse of the LAF₁ strain, that had been irradiated at approximately 7 weeks of age with 475r over the entire body. The left adrenal of the animal was removed 5 weeks following irradiation. This was one of a large

*Supported by grants from the Public Health Foundation for Cancer and Blood Pressure Research and the U. S. Public Health Service.

series in which groups of approximately 100 animals were subjected to similar though smaller doses of total or partial body irradiation with or without removal of adrenals or gonads. The data so far do not indicate any relationship between the treatment, including irradiation, and the mast cell tumor. However, as a thorough histologic study of the various neoplasms occurring in this series (over 2000 mice) has not yet been made, the question remains whether or not mastocytomas are induced at a low rate by the procedures to which this animal was subjected.

The animal was killed when a tumor measuring 15 x 20 x 22 mm was discovered in the subcutaneous tissue of the right side of the chest. The tumor was uniformly pale gray, did not invade the overlying skin and did not metastasize. The animal was slightly obese. The thymus was atrophic. The spleen measured 22 x 7 mm in the 2 greatest lengths. The right adrenal was slightly enlarged and yellow. The other endocrine glands appeared normal save for the ovaries; the right was the site of a 4 mm sized tumor, predominantly a luteoma, and the left was atrophic. There was a minute adenoma in the lung.

Transplantation. The original tumor was transplanted into 11 male mice of the strain of origin. The tumors became palpable in all animals 4 months after transplantation and grew slowly but continuously. They measured approximately 4 cm in diameter at the time of death 7 to 10 months following transplantation. Before study of the sections, the tumor, because of its site of origin, was presumed to be a mammary tumor; consequently to test its hormonal dependency the testes of 5 tumor-bearing hosts were removed. This procedure in no way altered the rate of growth of the tumor. There was a conspicuous absence of metastases and lack of necrosis even though the tumor masses weighed as much as 8 to 10 g each. There was also a remarkable lack of secondary effect on the host. Even the thymus, indicator of stress, was only slightly or moderately atrophic in the animals with smaller tumors which were sacrificed for transplantation. Testes, seminal vesicles and endocrine organs, including the adrenals, appeared normal. There has been

no change in the features of this tumor in the course of subsequent transplantations. Thus far there has been no failure to take and no tendency for the tumor to assume malignant features.

The second generation grafts were made in 2 passages on 14 female and 7 male mice all with success. Seven females received subcutaneous pellets of 7 to 8 mg diethylstilbesterol (containing diethylstilbesterol and cholesterol in the proportion 1 to 9). This treatment, given at a time when the tumor was thought to be of mammary origin, had no effect on the growth of the tumor. The first successive passages now in progress do not indicate any basic change in the features of the tumor.

All tumors were well circumscribed but invaded muscle tissue. Microscopically the only cells observed were mast cells. They contained varying numbers of granules of different sizes, which could be deeply stained with toluidine blue or Giemsa stain (Figs. 1,2). Granulation of the cells was as heavy as in normal tissue mast cells, from which they were indistinguishable. Microscopic examination of the draining lymph nodes disclosed no other change than the presence of a few normal mast cells (Fig. 3).

Assays. For determining the histamine, heparin and 5-hydroxytryptamine content of the tumors a weighed piece of tissue was minced finely with scissors and homogenized in water or isotonic sucrose solution with a glass tissue grinder until no fragments of tissue settled on standing for several minutes. *Histamine determination:* An equal volume of normal hydrochloric acid was mixed with a portion of the homogenate and the precipitated protein was centrifuged down. The supernatant was neutralized with normal sodium hydroxide and diluted with Locke solution. The extract was assayed for its histamine content using the isolated terminal ileum of the guinea pig suspended in Locke solution containing 6 mg of atropine per liter. *Heparin assay.* A solution of trypsin in phosphate buffer at pH 8 was added to a portion of the homogenate. This was incubated at 38° for 1½ to 2 hours. After incubation an equal volume of normal sodium hydroxide was added

to the solution. It was then neutralized and assayed for its heparin activity by determining at what dilution it would inhibit clotting in the *in vitro* system described below. Globu-

lin was prepared by adding an equal volume of saturated ammonium sulphate to fresh, oxalated dog plasma. The precipitate obtained by centrifugation was dialysed for 24

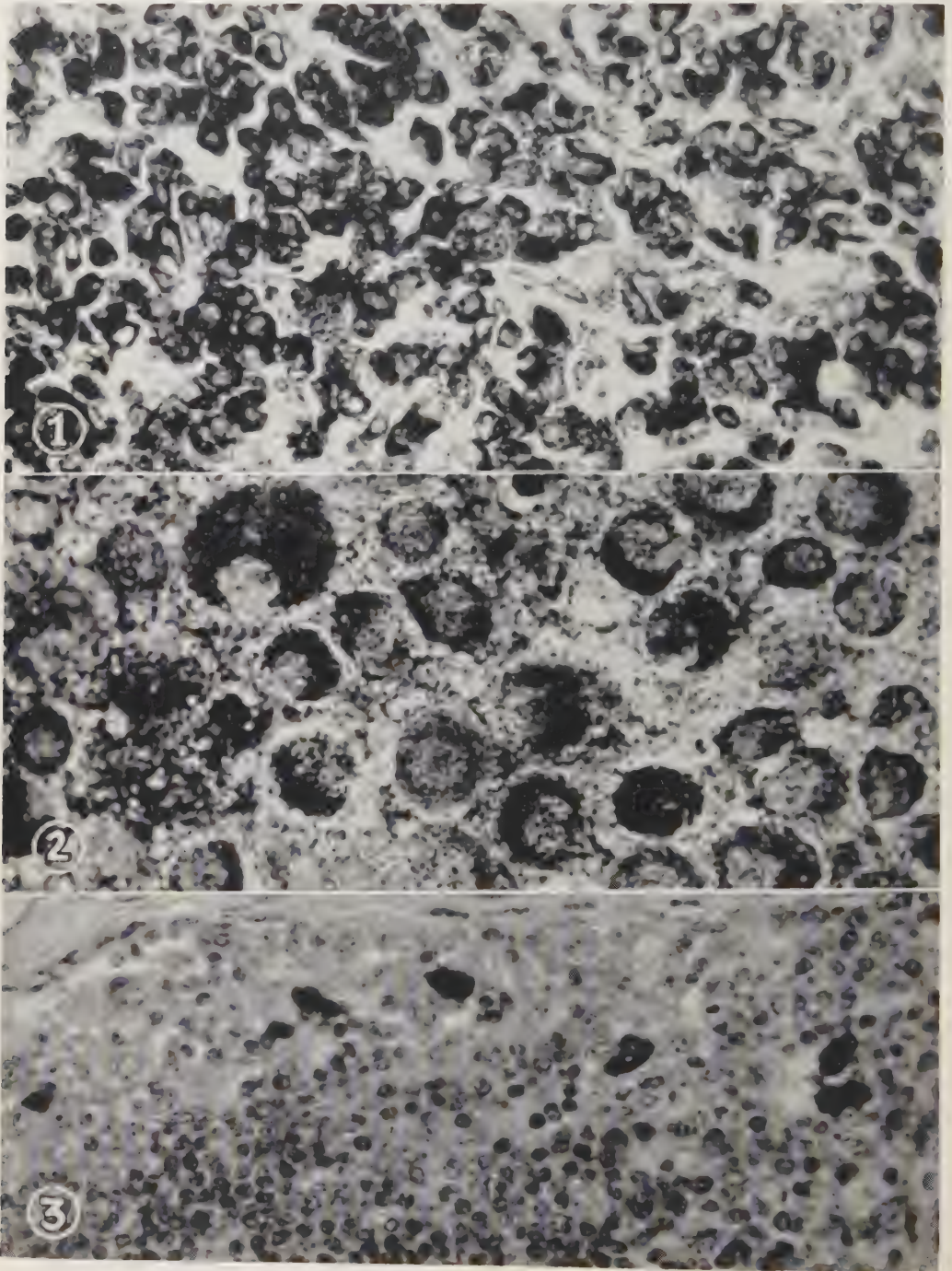


FIG. 1. Section of tumor cells stained with Giemsa stain. $\times 500$.

FIG. 2. Imprint of mastocytoma stained with toluidine blue. $\times 1000$.

FIG. 3. Section of a slightly enlarged lymph node in drainage region of a mastocytoma stained with Giemsa stain. $\times 370$.

hours with frequent changes of distilled water. Thrombokinas solution was prepared as a watery extract of acetone dried powder of rat brain. By trial and error it was found that 0.3 ml of globulin solution + 0.3 ml of thrombokinas solution + 1.0 ml water + 0.05 ml of a 5% calcium chloride solution gave a firm clot within 3 minutes at 37°. The formation of this clot could be inhibited by 2 I.U. of heparin but not by one unit.

5-Hydroxytryptamine detection and estimation. Perchloric acid was added to a portion of the homogenate to give a perchloric acid concentration of 0.4 N. The protein was eliminated by centrifugation. Potassium hydroxide was added to the supernatant until the pH was 7.5. The potassium perchlorate was thrown down by centrifugation and the supernatant was applied to a column of Amberlite XE96 cation exchange resin in the sodium form. The column was washed several times with distilled water. The amines were eluted with 2 N hydrochloric acid. The eluate was neutralized with sodium hydroxide and added to excess acetone to make a solution of 95% acetone with 5% water. The insoluble inorganic salts were removed by filtration and the 95% acetone filtrate evaporated to dryness. The dry residue was dissolved in approximately 0.5 ml of water. This watery extract was applied at several spots 3 cm from the edge of a large sheet of Whatman No. 1 filter paper for paper chromatography. Standard histamine and 5-hydroxytryptamine solutions were also applied to the starting line 3 cm from the edge of the paper. The solvent for chromatography was butanol acetic acid and water. After 12 to 15 hours of ascending chromatography the paper was dried. A strip corresponding to point of application of one portion of the extract was cut out and the rest of the paper sprayed with a solution of 0.2% ninhydrin in acetone containing glacial acetic acid(9). After heating the paper for 2 minutes at 100° a very large blue spot due to histamine was seen close to the starting line. A brown spot was seen about half way up the paper. These spots corresponded in position to the histamine and 5-HT standards. In addition, the 5-HT spots showed the characteristic fluorescence of 5-HT when examined under UV light

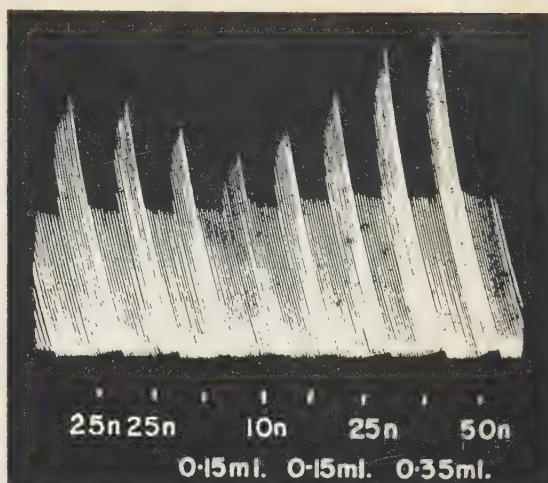


FIG. 4. Portion of 5-HT assay on isolated clam heart of material from 5-HT position of chromatogram from Exp. 6 extracted with 250 ml water. Upper line shows addition to the bath of 25, 10, and 50 μ g of 5-HT; lower line shows the addition of 0.15 and 0.35 ml of the sample.

(9). The piece of the non-sprayed paper corresponding to the 5-HT position on the sprayed paper was removed and extracted in distilled water. After appropriate dilution with sea water this solution was assayed for its 5-HT content on the isolated clam heart (*Venus mercenaria*) suspended in sea water (Fig. 4).

Results. The histamine, heparin and 5-hydroxytryptamine contents of several tumors are shown in Table I.

Discussion. The quantities of heparin and histamine present in these tumors are about twice those found by Cass *et al.* in spontaneous mastocytomas in dogs(10). According to Riley(11) 1.29 mg/g was the highest value ever recorded for tissue histamine. With one exception all values for our mastocytoma are higher than this. The presence of 5-hydroxy-

TABLE I. Histamine, Heparin and 5-HT Content of Mouse Mastocytomas.

Animal No.	Histamine, mg/g	Heparin, units/g	5-HT, μ g/g
1	.85	790	8
2	—	1540	—
3	3.36	247	*
4	2.75	249	—
5	4.2	500	—
6	3.3	660	140

A dash indicates that no determination was made.

* Detected but not quantitated.

tryptamine in mast cells, although not in mastocytoma, has been previously reported (3,12). The value 140 $\mu\text{g/g}$ in our mastocytoma is the highest recorded in mammalian tissues apart from carcinoid tumors, in which values up to twenty times greater have been found.

Summary. 1. A transplantable mastocytoma of the mouse is described. The tumor is benign and composed of cells indistinguishable from normal mast cells. 2. It contains greater quantities of histamine and heparin than have previously been found in normal or tumorous mast cells. It also contains large amounts of 5-hydroxytryptamine.

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Received July 3, 1957. P.S.E.B.M., 1957, v95.

Eggs of Floodwater Mosquitoes (*Diptera: culicidae*) VI. Effect of Metabolites on Latent Embryos in Uncapped Eggs.* (23376)

WILLIAM R. HORSFALL, L. M. HENDERSON[†] AND PATRICK T. M. LUM[‡]
(Introduced by L. M. Henderson)

Departments of Entomology and Chemistry, University of Illinois, Urbana

Latent embryos in intact eggs of floodwater mosquitoes become activated after a series of events called *conditioning*(1). Such embryos hatch quickly and uniformly when subjected to a reduction in oxygen in the medium surrounding the eggs. Reduction in oxygen is effected in nature by metabolism of embryos when eggs are crowded into closely confined sites beneath layers of leaves, or by metabolic activity of microbes dispersed among the plant debris. It has been accomplished in the laboratory by crowding(2), by microbes(3) by reducing agents(1,3,4,5) and by replacing the dissolved oxygen in the medium with nitrogen or other inert gases(3,6).

Activation of conditioned embryos in intact eggs is prompt when oxygen in the me-

dium is reduced to the proper level and at the proper rate. This fact suggests that latency is terminated by a sudden alteration in metabolic activity of the embryo. In preliminary experiments involving the effect of inhibitors of respiration on hatching of the eggs of aedine mosquitoes, the chorion proved to be impervious to solutes. Compounds which were quickly lethal to free larvae did not kill embryos in the intact eggs, even after exposure to these agents for several days. On the other hand, embryos in eggs made permeable by brief exposure to solutions of sodium hypochlorite were killed by the same inhibitors. Because of the barrier effect of the shell, latent embryos deprived of their shells or parts of shells were used in the experiments reported here.

Methods. All experiments cited herein were performed with embryos of *Aedes trivittatus* (Coq.) This species is well suited to experimentation because it (a) is easily conditioned, (b) is readily obtained in large num-

* Research supported by grants from the Division of Research Grants, U. S. Public Health Service.

[†] Present address: Dept. of Agricultural Chemistry, Oklahoma State Univ., Stillwater.

[‡] Present address: Entomological Research Center, Vero Beach, Fla.

bers, (c) is adapted to storage in the laboratory, and (d) is readily uncapped mechanically. Latent embryos used in the experiments were obtained from eggs deposited in the laboratory. Wild-caught females were caged, fed human blood and maintained at 25°C. Eggs deposited on moist cheesecloth bats were removed daily to moist filter paper and incubated in chambers saturated with water vapor until embryos were mature. They were then stored at 4°C. Mechanical uncapping was performed routinely on eggs removed directly from storage at 4°. Caps were removed mechanically from eggs by bending them at the normal circular line of fracture near the anterior end. Essential tools consisted of a pair of finely pointed forceps and a thin-walled, tapered, glass tube (breaking tube) having an inside diameter of such size as to fit snugly over an egg at the fracture line. The breaking tube was made by drawing out a piece of standard, melting-point, capillary tubing (1.5 x 2.0 x 90 mm) in a low flame. It was drawn so that the diameter at the end was smaller than that finally desired. It was shortened to the correct inside, apical diameter by snipping off the end with the forceps. This resulted in a tube with sharp or even jagged edges at the apex which was more effective than a smooth one. The inside diameter of a tube suitable for uncapping eggs of *A. trivittatus* or *A. sollicitans* should be 150-160 μ in diameter while one for *A. stimulans* should be 200-210 μ .

In practice eggs were uncapped while they were submerged in aqueous solutions. A standard, flat-form, porcelain, evaporating dish was a convenient container because it allowed more latitude for movement of tools. Eggs to be uncapped were wedged between the tips of the forceps. The breaking tube was placed over the anterior end of an egg, then was rocked gently until the cap was free along the fracture line. A stereomicroscope providing a magnification of about 10 diameters was ample for the dissection.

The medium (modified Ringer's solution) in which activation of exposed embryos was observed consisted of a nearly isotonic solution of salts. This solution contained 6.790 g of NaCl, 0.153 g of KCl, 0.153 g of CaCl₂,

and 0.120 g of NaHCO₃ per liter of distilled water. Metabolites to be tested for effect on latency were added to this solution and were neutralized with NaOH to pH 7.0.

This procedure for uncapping eggs and activating the embryos has been used in routine rearing of both univoltine and multivoltine species. Many species respond variously to hatching stimuli applied to intact eggs because the degree of conditioning is not uniform. Embryos when removed from their shells and placed in the modified Ringer's solution become activated and develop normally. This procedure has been particularly useful in work with the species having only one generation each year.

All experiments were done with the exposed embryos which had been stored for 24 hours in a test solution of each metabolite. By this means variation in permeability was eliminated. Each group of embryos which was stored in its own test solution was then divided into a control group and an experimental one. The control group was placed in Ringer's solution, and the experimental one remained in the solution of the metabolite dissolved in Ringer's solution. Both groups were kept at room temperature and observations were made of the number of active embryos at periodic intervals.

Results. The objective in developing this procedure for uncapping eggs was to place the embryos in more intimate contact with test media. It was hoped that procedures such as lowering of the oxygen tension or interference with glycolysis or respiration might cause termination of latency. If such were the case, it might be possible to ascertain the mechanism whereby the lowered oxygen tension of the medium stimulates hatching of intact eggs.

When embryos were exposed as described above and placed in an isotonic solution, they became active, normal larvae without further treatment. This behavior prompted a change from a study of the activation of embryos to a study of the factors concerned in maintaining latency. Addition of glucose or one of several other recognized sources of energy to the freshly exposed embryos prevented activation at 4°C. After a suitable period of time at this low temperature, embryos could

TABLE I. Effect of Metabolites on Latency of Conditioned Embryos of *Aedes trivittatus* 5 Months Old in Uncapped Eggs in the Laboratory, 1956.

Metabolite in Ringer's solution		No. embryos treated	Embryos activated in 48 hr	
Name	Conc.		No.	%
Glucose	.3M*	60	3	5
Fructose	.3M	30	5	16
Mannose	.3M	30	8	24
Inositol	.3M	30	14	46
Mannitol	.3M	30	20	66
Sorbitol	.3M	30	23	76
Galactose	.3M	30	26	80
Pyruvate	.3M	30	0	0
Glutamate	.1M	30	2	6
Acetate	.3M*	30	3	10
Citrate	.3M	30	3	10
Fumarate	.1M†	30	3	10
Malate	.1M†	30	10	33
Succinate	.1M	30	lethal	
Gelatin	.5%	30	23	76
Bovine albumin	2.5%	30	27	90
None	—	60	55	92

* Media containing 0.1M permitted activation of 80% of embryos.

† Media containing 0.01M permitted activation of 100% of embryos. (Alpha keto glutarate at 0.01M permitted activation of 83% of embryos.)

be maintained latent at room temperature for at least 48 hours in solutions of glucose and other substrates. Embryos from eggs less than 5 months old required no more than 24 hours at 4° while those 8 months of age required much longer storage at low temperature before use in experiments at room temperature. Bucklin(7) found that latency was terminated and normal morphogenesis of eggs of grasshoppers took place in Ringer's solution.

TABLE II. Effect of Increasing Concentrations of Salts in Ringer's Solution on Activation of Conditioned, Latent Embryos of *Aedes trivittatus* in the Laboratory, 1956.

Concentration of salts	No. of embryos treated	Embryos activated in 48 hr	
		No.	%
Ringer's solution	60	55	92
2× Ringer's solution	60	56	93
3× " "	60	52	88
4× " "	60	46	82
5× " "	60	47	78
10× " "	60	44	73*

* Lethal within 24 hr.

Metabolites dissolved in Ringer's solution varied widely in their effect on latency (Table I). Among the sugars, glucose and fructose caused continuation of latency; galactose permitted activation to the same extent as Ringer's solution alone; the 3 sugar alcohols used had little effect. Salts of several organic acids which are recognized substrates in oxidative metabolism all maintained latency approximately as well as glucose at comparable molar concentrations.

Hypertonic solutions of the inorganic salts used permitted the termination of latency in the same manner as isotonic Ringer's solution. Concentrations as high as 5 times the isotonic level were not harmful, but at 10 times the isotonic concentration activated embryos died (Table II). None of the solutions containing metabolites shown in Table I was used at concentrations which would give an osmotic pressure as great as 5 times the isotonic level.

Cold embryos exposed in distilled water became elongated and died after activation. Embryos from eggs stored at room temperature for a few days prior to uncapping became active and survived in distilled water.

Summary. Latency in both intact and manually uncapped eggs of floodwater mosquitoes may be continued or terminated at will. *Conditioned* embryos in uncapped eggs become active in a modified Ringer's solution. Latency may be continued in such embryos by adding metabolites such as glucose, fructose and salts of organic acids which are recognized substrates in oxidative metabolism to the basic Ringer's solution.

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